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EXPERIMENTAL CELL RESEARCH

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THREE-DIMENSIONAL OBSERVATIONS ON THE INTRANUCLEAR STRUCTURE

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Received September 28, 1955

THE fine structure of the mammalian nucleus at interphase has long been a controversial subject. The failure to obtain convincing findings is probably due to its delicate and intricate characteristics. Recently, chromosomal threads associated with the nucleolus in the nucleus of the rat lymphoblast [8] and chromosome structural details in the nucleus of the rat myeloid cell [3] were demonstrated using technical modifications destined to swell and flatten the cells freely liberated in suspension fluid. The procedures, however, inevitably resulted in distortion of the nuclear spheres and in displacement of the internal organized structures. The present study is an investigation of the undisturbed three-dimensional structure within the nuclear sphere and is correlated with the previous observations of the individual chromosomes.

OBSERVATIONS

In these investigations the mesothelial cells of the rat omentum were employed. These cells are flat and arranged in a single layer. The nuclei are thin, elliptic and with almost plane superior and inferior surfaces, each being covered externally by an extremely thin layer of non-granulated cytoplasm. These morphological characteristics make it possible to observe the intranuclear structures in the fresh state without any disrupting treatment, and also to demonstrate the clear-cut structures in successive slices of the nuclei, serially sectioned parallel to the flat surface.

A piece of omentum, 5 mm by 5 mm in size, was surgically removed from an anesthetized rat, rinsed in Ringer solution, stretched on a slide, and covered with a rectangular cover slip after which the edges were sealed with paraffin. The preparation was

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examined immediately by phase-contrast microscopy. Special care was taken to keep the preparation warm at all times. Nuclei lying with their flat surface perpendicular to the microscope axis were selected by focusing at different levels using the oil immersion lens.

When the flat surface of the nucleus was in focus, a network of dark, long, intertwining threads was observed extending throughout the entire area (Fig. 1, A_1). Each of the threads was recognized as a strand of minute dark granules serially arranged like beads on a string in those areas in sharp focus. When the middle plane or largest elliptic section of the same nucleus was in focus, the nucleolus was observed as a dark body almost in the center (Fig. 1, B_1). The threads were out of focus except those in the periphery and some on the nucleolus. The threads on the membrane were too densely distributed to distinguish their individual contour.

Following the above observations, stained sections of the same preparations were examined. The paraffin seal was removed from four corners of the cover slip. Carnoy's fluid was introduced from one corner by a micropipette as pieces of blotting paper were placed on the other corners, so that Ringer solution under the cover slip was gradually replaced by the fixative. Thus the tissue was fixed in the stretched state. After 12 hours the tissue was taken out and embedded in paraffin, placing it as evenly and horizontally as possible. Serial sections, one micron in thickness, were made parallel to the tissue surface and stained by the Feulgen method combined with light green staining.

The superficial slice of the nucleus of the mesothelial cell exhibited a network of long, intertwining, Feulgen-positive threads extending throughout

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Fig. 1. Photomicrographs of the interphase nuclei of rat omentum cells and their corresponding sketches.

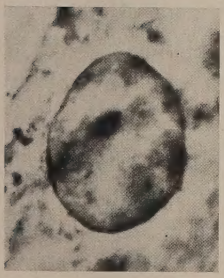
A_1 and B_1 are of the fresh cell, taken by a Leitz-Wetzlar Panphot phase-contrast microscope (Periplan 15× and Pv Apo Oel 90:1), focusing on the surface (A_1) and on the middle plane (B_1) of the same nucleus. A_1 demonstrates a network of dark, long, intertwining threads. A part of the thread which is sharply in focus at the lower region shows three dark granules serially arranged on the thread. B_1 shows the nucleolus almost in the center. The threads are generally out of focus except those on the membrane and some on the nucleolus.

A_2 and B_2, taken by a Leitz-Wetzlar Panphot microscope (Periplan 15× and 2 mm Apo Oel), are of two successive, longitudinal slices of the cell stained by Feulgen-light green staining. A_2 is the superficial slice of the nucleus which demonstrates a network of long intertwining, Feulgen-positive threads. B_2 is the slice of the middle layer of the same nucleus with its superior and inferior layers cut away. Feulgen-positive threads are distributed on and near the membrane except a few which are in the interior attaching to the nucleolus at their subterminal regions and radiating their free arms toward the periphery. Note that the intranuclear structures in A_1 and B_1 correspond respectively to those in A_2 and B_2.

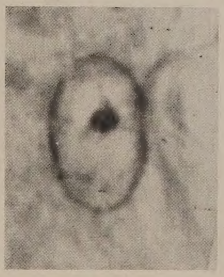
C_1 is an enlarged print of A_2. In the marked area, three Feulgen-positive granules are seen serially arranged on two parallel, faintly stained filaments. C_2, taken and printed at the same magnification as C_1, shows prophase stained by the Feulgen method after pretreatment with a dilute solution of ammonia. Feulgen-positive granules are serially arranged on two parallel chromonemata. Structural similarities are to be recognized by comparing C_1 and C_2.

A1

A2



B1



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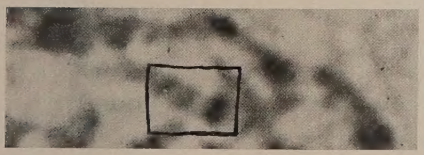
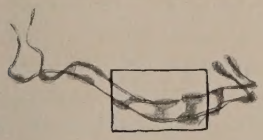
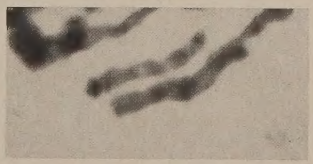
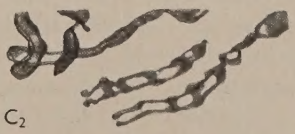




C1





C2

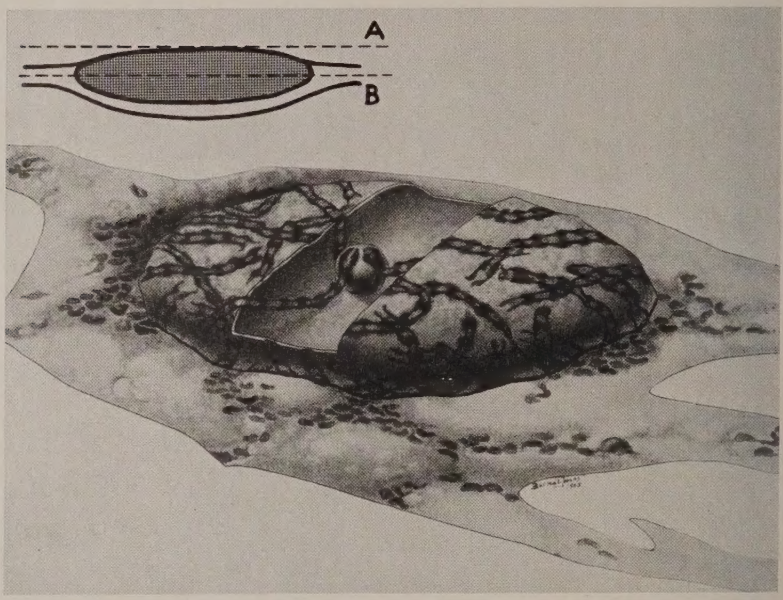


Fig. 2. Schematic drawing illustrating the three-dimensional structure of the nucleus of a mesothelial cell of the rat omentum. Reconstructed according to the findings described in the text.

the entire area (Fig. 1, A_2). Each of the threads consisted of deeply stained minute granules which were serially arranged on a pair of parallel, faintly Feulgen-positive filaments. By focusing sharply it was observed that each granule was transversely dumb-bell shaped, or composed of two bodies almost equal in size which were located on either filament and connected by a bridge (Fig. 1, C_1). The succeeding slice of the same nucleus which included only its middle layer with the superior and inferior layers cut away showed a definite distribution of the Feulgen-positive threads, many being on the inner surface of the nuclear membrane and several radiating from the nucleolus and at their subterminal regions attached to the latter (Fig. 1, B_2).

According to the above findings, the three-dimensional structure within the nucleus was reconstructed as shown schematically in Fig. 2.

An additional observation was carried out to study the fine structure of the mitotic chromosomes in comparison to that of the Feulgen-positive threads in the interphase nucleus. The preparation of the rat omentum was treated with a 3 per cent solution of ammonia for 10 minutes prior to fixation and

Feulgen staining. The prophasic chromosomes of the mesothelial cell became stretched and were no longer uniformly Feulgen-positive (Fig. 1, C_2). Each one was composed of two parallel, faintly Feulgen-positive chromonemata on which transversely bridged pairs of densely Feulgen-positive granules were serially arranged at varied intervals, showing an extreme resemblance to the Feulgen-positive thread in the interphase nucleus.

DISCUSSION

From the observations described herein, it may be stated that the fresh nucleus is not structureless but a definite formed structure consisting of dark threads present within. The threads observed at the superficial and interior levels of the fresh nucleus were similar in form and distribution to the Feulgen-positive threads observed in serial sections of the nucleus. The Feulgen-positive threads, especially those in which the fine structure was distinctly observable, exhibited a close resemblance in structural details, in addition to stainability, to the stretched mitotic chromosomes. These comparisons suggest that the Feulgen-positive threads and, accordingly, the dark threads observed in the fresh nucleus are the chromosomes persisting in the interphase nucleus. Furthermore, it might be interpreted, according to Hughes' assumption [1], that the granules on the threads are unchanged segments of the mitotic chromosomes while the filaments are stretched chromonemata.

The distribution thus observed differs from a view that the chromosomes in an extended state fill the entire area of living nucleus unless they condense upon injury [9]. The present findings are supported by other reports. Hughes [2] and Ludford [6] concluded that the interphase chromosomes may line the inside of the nuclear membrane because the prophasic chromosomes were seen in this position. Furthermore, Ludford [6], on examining a superficial slice of the nucleus and a nucleus with the top of the membrane cut away in sections of mouse carcinoma tissues, stated that the chromosomal elements were distributed on the inside of the membrane and on the nucleolus in the interior.

Ludford [6], from the above observation, and Ris and Mirsky [10], on recognizing isolated chromosomal threads with attached nucleoli, suspected the possible existence of nucleolus-organizing regions on the particular chromosomal structures. Kinosita and Ohno [4], and Kinosita, Ohno, and Tanaka [5] demonstrated on some mitotic chromosomes of the rat myelocyte, a chromophobic segment resembling the secondary constriction or SAT-zone on the chromosome of plants and lower animals. This zone has been considered

as a nucleolar organizer. Ohno and Kinosita [7] showed that similar specific segments were present at the subterminal regions of three definite autosomes in the rat diploid lymphoblast. Further, Ohno and Kinosita [8] found that in the rat interphase lymphoblast three Feulgen-positive threads corresponding in comparative size to the three special autosomes were also attached to the nucleolus at their subterminal regions. These observations suggested that the threads associated with the nucleolus were the special chromosomes with the nucleolar organizers at their subterminal regions. The Feulgen-positive threads associated with the nucleolus, observed in the present study, likewise were several in number and attached to the nucleolus at their subterminal regions suggesting that they were the chromosomes with the nucleolar organizers from which the nucleolus developed.

SUMMARY

The interphase nuclei of living mesothelial cells of the rat omentum were examined on their superficial and interior levels. Then, comparative observations were undertaken on serial sections of the nuclei of these cells, prepared from the same preparation and stained by Feulgen-light green staining.

The nucleus contained threads which were dark in the fresh nucleus, Feulgen-positive in the stained nucleus, and each type was similar in form and distribution to the other. Their fine structure was comparable to that of stretched mitotic chromosomes. A few of them were attached at their subterminal regions to the nucleolus, corresponding to the special chromosomes with SAT-like zones, previously reported.

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THEORETICAL ASPECTS OF COMPETITIVE ANTAGONISM

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Since the work of Woods [23], the concept of competitive antagonism has developed rapidly, and a great many facts pertaining to it have been discovered. These are admirably summarized by Woolley in his recent book [25].

However, the most widely accepted hypothesis of the mechanism of competitive antagonism does not account for all the observed facts. Moreover, it is possible that certain discrepancies between theory and observation have been completely ignored. Because such discrepancies had been encountered in the course of experimental work [19], an attempt was made to perfect and extend the theory as described in this paper.

DERIVATION OF EQUATIONS FOR CHARACTERISING COMPETITIVE ANTAGONISM

A competitive antagonist is considered to produce its inhibitory effect by combining reversibly with the enzyme which metabolises the substrate it resembles; the enzyme-inhibitor complex so formed, unlike the enzyme-substrate complex, does not react further. From a consideration of the Mass Law equations of these two reactions, Shive and Macow [22] concluded that the ratio of concentration of inhibitor to concentration of substrate for any desired degree of inhibition, is constant; this ratio has been named the inhibition index. As applied to biological systems such as growing organisms, the inhibition index is derived from the concentrations of inhibitor and substrate in the external medium.

It will be shown here that the inhibition index is not constant. The mathematical treatment is based on the laws governing reactions in heterogeneous systems. Reasons for this will be considered later.

The fundamental postulate is that the substrate (metabolite) in combining with the enzyme follows the laws of adsorption as formulated by Langmuir [8]; the inhibitor (antagonist) is adsorbed on the enzyme in exactly the same

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manner. The enzyme-substrate complex decomposes in two ways, by reversion to enzyme and substrate, and by conversion to enzyme and products, the rates of both these processes being proportional to the fraction of the enzyme surface occupied by substrate; the enzyme-inhibitor complex decomposes only by reversion to enzyme and inhibitor, the rate being proportional to the fraction of the enzyme surface occupied by inhibitor. When a steady state has been reached the rates of formation of enzyme-substrate complex and enzyme-inhibitor complex exactly balance their rates of decomposition, and the following equations hold:

$$k_1 S (1-a-b) = a (k_2 + k_3) \tag{1}$$

$$k_4 I (1-a-b) = b k_5. \tag{2}$$

k_1, k_2, k_3, k_4, and k_5 are specific rate constants, S and I are the concentrations of substrate and inhibitor respectively, and a and b are the fractions of the total enzyme surface occupied by substrate and inhibitor respectively.

A straightforward mathematical solution of these equations gives

$$a = \frac{PS}{QI + PS + 1} \tag{3}$$

and

$$b = \frac{QI}{QI + PS + 1} \tag{4}$$

where P and Q are $k_1/(k_2+k_3)$ and k_4/k_5 respectively.

Rearrangement of equation (3) gives

$$aQI = S(P - aP) - a \tag{5}$$

from which two more equations may be derived:

$$\frac{I}{S} = \frac{P - aP}{aQ} - \frac{1}{Q} \cdot \frac{1}{S} \tag{6}$$

and

$$\frac{S}{I} = \frac{aQ}{P - aP} + \frac{a}{P - aP} \cdot \frac{1}{I}. \tag{7}$$

Similarly from equation (4) the following may be derived,

$$\frac{I}{S} = \frac{bP}{Q - bQ} + \frac{b}{Q - bQ} \cdot \frac{1}{S} \tag{8}$$

and

$$\frac{S}{I} = \frac{Q - bQ}{bP} - \frac{1}{P} \cdot \frac{1}{I} \tag{9}$$

These equations may be applied to the study of growth inhibition in living organisms. Experimental conditions are so designed that the only variables are the concentrations of metabolite and inhibitor, and the growth response of the organism. Under these conditions, when the rate of the inhibited reaction is the growth-controlling factor, the rate of growth is determined by the magnitude of a. To obtain a defined rate of growth or a defined amount of growth after a constant interval of time the value of a must be constant.

When a is constant, the inhibition index, I/S, is a linear function of the reciprocal of the substrate concentration. The reciprocal of the inhibition index, which may be termed the "metabolic index", is a linear function of the reciprocal of the concentration of antagonist. It should be noted that as the concentrations of substrate and inhibitor are raised the second term in equations (6) and (7) approaches zero. At high concentrations the second term would be negligible compared with the first, and the inhibition index would be substantially constant.

Although straight lines are expected when experimental data are plotted according to equations (6) and (7), these results may be modified by factors not taken into account in the derivation of the equations. For example, if the inhibitor is destroyed or converted into an inactive product, the concentration of inhibitor may alter considerably during the course of the experiment. This may be reflected in the plotted results by curved instead of straight lines. Thus when I/S is plotted against $1/S$ the line would curve away from the $1/S$ axis as $1/S$ increased; when S/I is plotted against $1/I$ the line would curve away from the S/I axis.

Equations identical with (6) and (7) can be obtained if the reactions are treated as homogeneous. The reason for the failure of Shive and Macow [22] to arrive at these equations is their failure to consider the equilibrium conditions of all three reactants, enzyme, substrate and inhibitor, instead of the equilibrium conditions of the separate reactions of enzyme with substrate and enzyme with inhibitor.

From the preceding paragraph it would seem that there is no particular merit in basing the mathematical treatment of competitive antagonism on the laws of heterogeneous reactions. However, this approach, rather than that of homogeneous reactions, was chosen for the following reasons.

(*a*) It clearly shows that the equations are applicable to reactions other than enzyme reactions.

(*b*) It provides a more satisfactory foundation for the explanation of growth stimulation by low concentrations of inhibitors (see subsequent section).

(*c*) Recent studies [3, 5, 10] have shown that many intracellular enzymes are associated with insoluble particles, so that many intracellular enzyme reactions must be heterogeneous.

It should, perhaps, be pointed out that current ideas on the nature of the enzyme-substrate complex need not be changed. Chemisorption may easily be distinguished from physical adsorption [2], but it is not easily distinguished from compound formation [2, 8], the differences here being quantitative rather than qualitative.

Evaluation of the Fraction of the Total Enzyme Surface Occupied by Substrate

It has been postulated above that the rate of growth is proportional to the fraction of the enzyme surface occupied by substrate for the growth-limiting enzyme reaction. The actual numerical value of this fraction is not known, so far, for any enzyme, and it is not necessarily true that the fraction will be one half when the rate of growth is one half the normal rate. In fact, in attempting to explain growth stimulation by inhibitors (see subsequent section), it has been necessary to postulate that even in the normally-functioning cell the value of this fraction is small, probably less than one half. A method of evaluating this fraction would, therefore, be useful.

When a in equation (6) is constant, the term $(P-aP)/aQ$ becomes constant and we may write

$$\frac{P-aP}{aQ}=C. \tag{10}$$

The rate of growth, G, is proportional to a.

Therefore, $$G=na \tag{11}$$

and $$a=G/n \tag{12}$$

where $n=a$ constant.

Substituting G/n for a in equation (10), and rearranging, we get

$$\frac{nP-GP}{GQ}=C. \tag{13}$$

If experimental data are plotted according to equation (6) at two different levels of growth or growth rates (i.e. at two different but constant values of a), two parallel straight lines should result. On extrapolation to cut the I/S axis, two values for C (equations (10) and (13)) are obtained. The two

values of G are known, and the value of Q is obtained from the slope of the lines. Substitution of these known values in equation (13) gives two simultaneous equations which may be solved for P and n. From the value of n may be calculated (equation (11)) the two values of a corresponding to the two levels of growth or growth rates. The values of a and G may then be plotted against each other, and a straight line drawn through the points. An extrapolation to the value of G in uninhibited tissue gives the normal value of a.

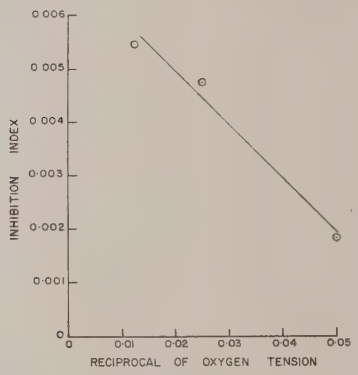
APPLICATION OF THE EQUATIONS TO THE RESULTS OF ANTIMETABOLITE STUDIES

Woolley [25] has recognised the antagonism between carbon monoxide and oxygen for haemoglobin as an instance of competitive antagonism, and this, being one of the best known and simplest examples of the phenomenon, deserves consideration first. Here the reactions are non-enzymic, the reactant gases being merely adsorbed on the haemoglobin, and equations (6) and (7) should apply. The results given in Fig. 65 of Peters and Van Slyke [18] were used. After the necessary extrapolation of the curve at 80 mm Hg oxygen tension was made, the inhibition index at 40 per cent oxyhaemoglobin was calculated for each of the 3 curves (at 80, 40 and 20 mm Hg oxygen tension) and plotted against the reciprocal of the oxygen tension. The results in Fig. 1 show that a straight line of negative slope gives a fair interpretation, in agreement with theory.

Some results with living organisms will now be considered. In the following examples, results presented in the original publications in tabular form were first plotted to give the typical family of curves so that the concentrations of inhibitor and metabolite at a suitable level of growth could be read off. These values were plotted against one another, a straight line graph fitted to the points (equation (5)) and the value of the metabolite concentration read off at the concentration of inhibitor required to reduce the growth to the same chosen level in the absence of the metabolite. This value represents the amount of metabolite produced endogenously and can be used to correct the values of the metabolite concentration in equations (6) and (7). In the examples considered immediately below, the correction was negligibly small. Consequently, the values of the reciprocal of the inhibition index at a suitable level of growth were calculated from uncorrected data and plotted against the reciprocal of the inhibitor concentration according to equation (7). It was hoped in this way to avoid the negative slope predicted by equation (6).

Data from three publications [6, 9, 21] were plotted in this manner, and the result in every case was, contrary to expectation, a straight line of negative slope. An example for the antagonism between norleucine and methionine [6] is given in Fig. 2.

The remarkable feature of these results is that the straight line seems to conform to equation (9) instead of equation (7), and if this be true, the value



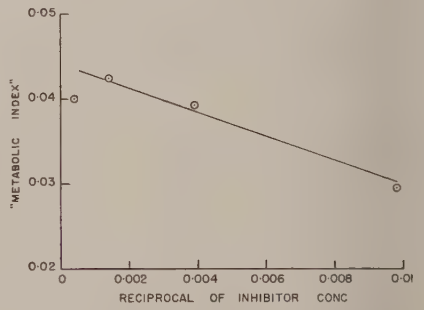


Fig. 1 (*left*). The relationship between the inhibition index and the reciprocal of the oxygen tension for the competition between carbon monoxide and oxygen for haemoglobin (from the data of Peters and Van Slyke [18]).
Fig. 2 (*right*). The relationship between the "metabolic index" and the reciprocal of the inhibitor concentration for growth inhibition of *E. coli* by norleucine and its antagonism by methione. (Calc. from data of Harding and Shive [6].)

of b must be constant. Therefore it appears that the fraction of the enzyme surface occupied by the antagonist, rather than that occupied by substrate, is the factor determining the rate of growth. This anomaly can be explained if the apparent competitive antagonist is assumed to undergo the same metabolic reaction as does the substrate, the true inhibitor being the product of this reaction or of some subsequent reaction. It must be assumed, further, that the inhibitor which is produced inhibits in a non-competitive manner, its effect being strictly proportional to the concentration, the inhibition being produced in all probability, by an irreversible combination of the inhibitor with an enzyme metabolizing the normal product. This assumption is necessary for the following reason: If the fraction of the enzyme surface occupied by "antagonist" is the growth-limiting factor, then the real inhibitor is produced at a constant rate when the growth rate is constant. This inhibitor can have a constant effect only if it acts non-competitively. In fact, it can be

shown by mathematical procedures such as used above in the derivation of equations (6) and (7), that if the antimetabolite gives rise to an inhibitor which acts competitively, this case cannot be distinguished from the simple one by plotting the results as described.

Support for the first of these assumptions comes from the work of several investigators; the antimetabolites fluoroacetate [16, 17], desoxypyridoxine [25], ethionine [25], 8-azaguanine [7, 13], 2-thiouracil [11] and 5-bromouracil [4, 26] have all been shown to undergo the same reactions as their corresponding metabolites in certain organisms. The conversion of the antimetabolite into the real inhibitor must, therefore, be a common phenomenon.

No instance of the irreversible combination of antimetabolite with enzyme is known to the author, but, in support of the second assumption, is the fact that the combination of biotin with avidin is irreversible [25] and so is the combination of apo-enzyme and co-enzyme of the aspartic-glutamic transaminase [25].

Thus, two types of competitive antagonism are postulated, the first being a true antagonism, the second an apparent one. In the first type the antimetabolite combines reversibly with the enzyme metabolizing the substrate it resembles; in the second type the "antimetabolite" undergoes the same reaction as the substrate with the formation of an inhibitory product, which then combines irreversibly with the enzyme metabolizing the normal product. When I/S is plotted against $1/S$, the first type gives a straight line of negative slope (equation (6)), whereas the second type gives a straight line of positive slope (equation (8)). When S/I is plotted against $1/I$, the first type gives a straight line of positive slope (equation (7)) whereas the second type gives a straight line of negative slope (equation (9)).

Before accepting these ideas as being of general application, it must be shown that the equations adequately describe the experimental findings in at least a few instances in which the behaviour of the inhibitor is known. Only for a very few reactions is this information available and, for many of these, the data are too incomplete to allow the necessary graphs to be plotted. In the following examples, possible confusion might have been avoided by using either equation (6) or equation (7) throughout, but this was inconvenient because of the varied methods of presentation of the results in the original publications.

Competitive antagonism between metallic ions provides a very good test case. From the figures of MacLeod and Snell [14] for the antagonism between sodium and potassium ions in various bacteria, were calculated the reciprocals of the inhibition indices and the reciprocals of the inhibitor

concentrations. If for each organism there is a true competitive antagonism, i.e. antagonism of the first type, the graph showing the relationship between these two variables should be a straight line of positive slope as indicated by equation (7). The plotted results presented in Fig. 3 show that this is so.

The antagonism between pyrithiamine and thiamine will now be considered. In the living organism and in isolated enzyme systems thiamine is

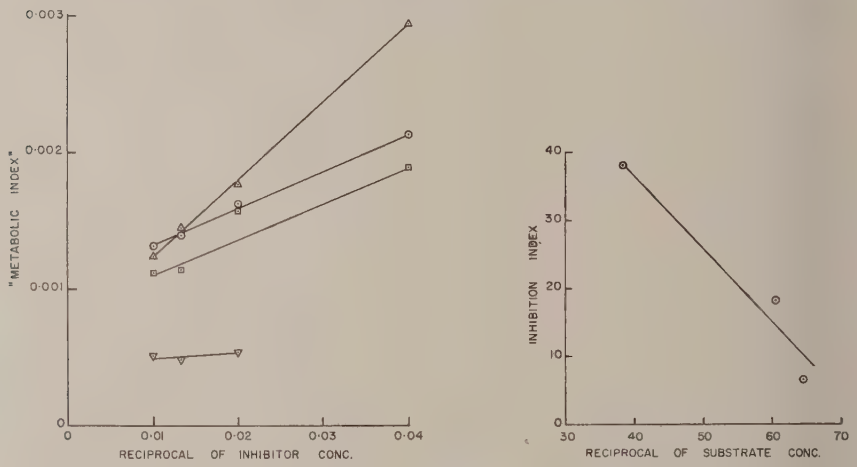


Fig. 3 (*left*). The relationship between the "metabolic index" and the reciprocal of the inhibitor (Na^+) concentration for the antagonism between sodium and potassium ions in bacteria. (From the data of MacLeod and Snell [14].) △—△ *S. faecalis*; ⊙—⊙ *L. arabinosus*; ⊡—⊡ *L. casei*; ▽—▽ *L. mesenteoides*.

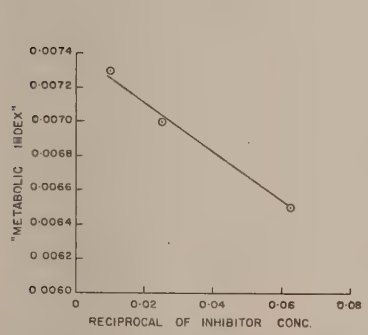
Fig. 4 (*right*). The relationship between the inhibition index and the reciprocal of the substrate concentration for growth inhibition of *L. fermentum* by pyrithiamine and its antagonism by thiamine (from the data of Sarett and Cheldelin [20]).

converted into thiamine pyrophosphate which is a co-enzyme for carboxylase. Pyrithiamine has been shown to be a competitive antagonist of thiamine; it does not compete with thiamine pyrophosphate [25]. The evidence is very strongly in favour of a direct competition between pyrithiamine and thiamine without any transformation of the pyrithiamine into its pyrophosphate analogue.

Unfortunately, Woolley's [24] results are not complete enough to allow plotting the necessary graphs for this particular case of antagonism. The results of Sarett and Cheldelin [20] are also not as complete as may be desired but they allow the necessary graphs to be plotted. These authors studied

the inhibitory effects of pyrithiamine on the growth of *Lactobacillus fermentum* and *Penicillium digitatum*. It is assumed that the effects are the same in bacteria as in chicken blood, which was studied by Woolley.

From the results for *L. fermentum*, corrected values for the inhibition index and the reciprocal of the metabolite concentration were obtained and plotted against one another. Although the results (Fig. 4) are too few in number to



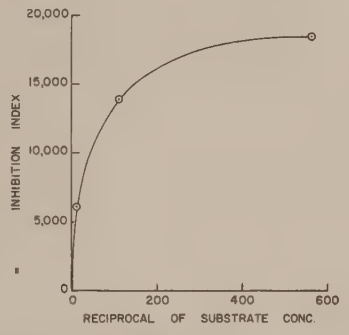


Fig. 5 (*left*)). The relationship between the "metabolic index" and the reciprocal of the inhibitor concentration for growth inhibition of *L. casei* by 5-bromouracil and its antagonism by thymine (from the data of Hitchings *et al.* [9]).
Fig. 6 (*right*). The relationship between the inhibition index and the reciprocal of the substrate concentration for growth inhibition of *Enterococcus* W12 and its antagonism by *p*-aminobenzoic acid (from the data of Aughagen [1]).

show definitely whether the graph is a curve or a straight line, there can be no doubt that the inhibition index increases with increasing concentrations of substrate—a feature of true competitive antagonism.

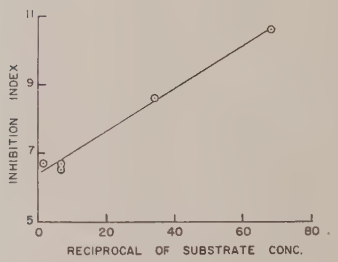
In passing it may be mentioned that the pyrophosphate analogues of thiamine and pyrithiamine give similar results [19].

The above examples refer to real competitive antagonism. As an example of the second type, the antagonism between 5-bromouracil and thymine in *Lactobacillus casei* is suitable. It has recently been shown that 5-bromouracil is incorporated into the deoxyribonucleic acid of *E. coli*, *Streptococcus faecalis* and others [4, 26]; its behaviour in *L. casei* is assumed to be the same.

From the results of Hitchings *et al.* ([9], Fig. 4) values for the metabolic index and the reciprocal of the inhibitor concentrations were calculated and plotted against one another. According to the theory the graph should be a straight line of negative slope; Fig. 5 shows that the results conform closely to this.

The Mode of Action of Sulphanilamide

Sulphanilamide was the first compound to be recognised as a competitive antagonist, and yet, its mode of action has not been completely elucidated after fifteen years of intensive research. The application of the equations developed in a previous section may help to throw some light on this problem.



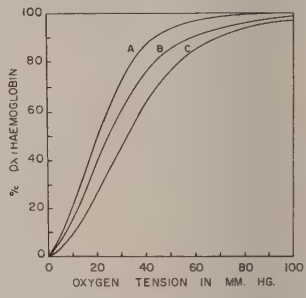


Fig. 7 (*left*). The relationship between the inhibition index and the reciprocal of the substrate concentration for growth inhibition of flax seedlings by sulphanilamide and its antagonism by *p*-aminobenzoic acid (from the data of Moore [15]).
Fig. 8 (*right*). The relationship between oxygen tension and oxygen saturation in human blood at pH's 7.64 (*A*), 7.44 (*B*) and 7.24 (*C*). (Taken from Peters and Van Slyke [18].)

Two sets of results were selected, those of Auhagen [1] and of Moore [15]. In his paper, Auhagen gives curves showing the relationship between amount of growth and *p*-aminobenzoic acid concentration at different fixed concentrations of sulphanilamide for the organism *Enterococcus* W12. From these curves the inhibition index was calculated and plotted against the reciprocal of the *p*-aminobenzoic acid concentration. The result is shown in Fig. 6. The graph is not a straight line but the inhibition index falls with rising concentrations of *p*-aminobenzoic acid. This suggests that sulphanilamide is itself not inhibitory but is changed into an inhibitory compound.

From Moore's [15] results for the inhibition of growth in flax seedlings by means of sulphanilamide the reciprocals of the *p*-aminobenzoic acid concentrations were calculated and plotted against the inhibition indices as in Fig. 7.

A straight line fits the points very well. The line has a positive slope, again indicating that sulphanilamide undergoes the same reaction as the normal substrate, *p*-aminobenzoic acid, with the formation of an inhibitory product. It is known that *p*-aminobenzoic acid is incorporated into the folic acid

molecule. The suggestion is made, based on these results, that an analogue of folic acid containing sulphanilamide instead of *p*-aminobenzoic acid is formed and that this inhibits the reactions in which folic acid participates.

THE STIMULATORY EFFECTS OF INHIBITORS

Stimulation of growth in living organisms by low concentrations of inhibitors is a well-known phenomenon for which no entirely satisfactory explanation has been advanced. In all probability no single theory will be found to be adequate. Woolley [25] mentions two of the suggested explanations. Another explanation is now suggested.

In the following discussion, reference will be made to the competition between oxygen and carbon monoxide for haemoglobin. Fig. 8, reproduced from Fig. 52 of Peters and Van Slyke [18] shows the relationship between oxygen tension and the oxygen saturation of haemoglobin. It can be shown experimentally that the shapes of the curves would be exactly the same if the gas were carbon monoxide instead of oxygen with the difference that the pressures of carbon monoxide would be lower by a factor of 1/210. Then it can be assumed that the curves are valid also for a mixture of oxygen and carbon monoxide. The following conclusions can be drawn from the curve *C* of Fig. 8. The percentage of oxyhaemoglobin at 20 mm of oxygen is 26. If a quantity of carbon monoxide equivalent to 20 mm of oxygen were introduced into the system represented by the curve, the percentage of combined haemoglobin would be 63 per cent. But, according to the law relating the percentage of combined haemoglobin with the gas pressure, the percentages of oxyhaemoglobin and carbon monoxide haemoglobin are the same, i.e. 31.5 per cent. Thus the admission of a small quantity of carbon monoxide to the system containing 26 per cent of oxyhaemoglobin has resulted in an *increase* of the latter to 31.5 per cent. This remarkable effect results from the fact that the interaction of haemoglobin with oxygen is of such a nature that the curve showing the relationship between the oxygen pressure and the percentage of oxyhaemoglobin (the so-called oxygen-dissociation curve) is not hyperbolic but sigmoid.

The molecule of haemoglobin possesses four haemin groups at each of which a molecule of oxygen or carbon monoxide may be adsorbed. The deviation of the oxygen dissociation curve from the expected hyperbola is explained by assuming that the adsorption of a molecule of gas at one haemin group can influence the adsorption of another molecule at one of the remaining groups. This phenomenon is not unique in Nature; in fact, it may be regarded as a special instance of the so-called "co-operative effect", i.e. the facilitation of further adsorption by small quantities of substance already adsorbed. For instance, the adsorption of water vapour at 100° C on wood charcoal shows a curve very similar to curve *C* of Fig. 8 ([2], p. 176). In this case, two explanations are possible, one in terms of adsorption in multilayers and the other in terms of the co-operative effect.

The basis of the suggested explanation for the stimulatory effects of low concentrations of inhibitors on intracellular enzyme reactions is that the "substrate-dissociation curve", (i.e. the curve relating the concentration of enzyme-substrate

complex to the concentration of the substrate) is not hyperbolic but sigmoid. Discarding the suggestion of adsorption in multilayers as inapplicable to enzymes, a curve of this form could by obtained only when the adsorption of a substrate molecule at one enzyme centre is influenced by the presence of an already-adsorbed molecule on a nearby centre. For enzymes with only one active centre on each molecule, this is likely to happen only if the molecules are closely aggregated, and in view of the close association of intracellular enzymes with sub-cellular morphological units, such an aggregation seems very probable. If this be true, then it is easy to see how low concentrations of a competitive antagonist may increase the quantity of substrate adsorbed, in exactly the same manner as low concentrations of carbon monoxide increase the concentration of oxyhaemoglobin in the system described above. This provides a reasonable explanation for any observed increase in the rate of the reaction if, as postulated, the rate of the reaction is dependent upon the fraction of the enzyme surface covered by adsorbed substrate. In extending the explanation to growth stimulation in organisms, it must be assumed that the stimulated reaction is growth-limiting, or capable of influencing the growth-limiting reaction.

An explanation in terms of the Michaelis-Menten picture of enzymatic reactions would require the postulate that all the molecules of the enzyme are so linked together that the structure may be considered as a single gigantic molecule with many active centres, and that the combination of substrate with enzyme at one centre alters the dissociation constant of enzyme-substrate complex at a nearby centre. This is considered to provide a less satisfying picture than that based on adsorption.

The theory outlined above may be tested experimentally. As will be seen from curve *C* of Fig. 8, when the percentage of oxyhaemoglobin is about 60 per cent, an increase is not observed when carbon monoxide is introduced. This suggests that if the proposed explanation of growth stimulation is correct, the percentage of enzyme saturated with substrate is low, i.e. there is an excess of free enzyme. No experimental data are as yet available which give an indication of the percentage saturation of any enzyme in the living cell, but a method for estimating this has been described already (p. 578).

Another consequence of the theory is that in a series of inhibitors there should be a correlation between the magnitude of the stimulation and the rapidity with which inhibition develops with increasing concentrations of inhibitor. For an understanding of this, reference will be made once again to the systems consisting of haemoglobin and oxygen which are represented by the curves in Fig. 8.

An examination of this figure shows in analogy to the considerations on p. 585, that when the O_2 tension is 20 mm in the system represented by curve *A*, the carbon monoxide tension must be quadrupled in order to produce a decrease in the percentage of oxyhaemoglobin from a value just perceptibly below the starting level to one that is two thirds of this. Moreover this decrease is preceded by a very small rise (1 per cent) in the percentage of oxyhaemoglobin at carbon monoxide tensions lower than that required to produce a perceptible reduction. For system *C*, on the other hand, when the O_2 tension is 20 mm the carbon monoxide tension required to lower the percentage of oxyhaemoglobin to two thirds its starting value is less than double that required to cause a reduction which is just perceptible, whereas, at tensions insufficient to produce any reduction there is pronounced increase (6 per cent) in the percentage of oxyhaemoglobin.

The analogy between this example and an enzyme system in living organisms is apparent if the haemoglobin is considered to represent an enzyme, oxygen its substrate and carbon monoxide a competitive antagonist. If the substrate is adsorbed with little interaction between adsorbed molecules the situation would be represented by a curve such as curve *A* of Fig. 8, whereas if there were considerable interaction between adsorbed molecules the situation would be represented by a curve such as curve *C*. In the first case a competitive antagonist would produce very little stimulation at low concentrations and growth inhibition, once produced, would increase slowly with increase in the concentration of inhibitor. In the second case low concentrations of the inhibitor would produce marked stimulation of growth, and inhibition, once produced, would develop rapidly with increasing concentrations of inhibitor.

One possible objection to this theory of stimulation is the fact that additions of metabolite seldom duplicate the stimulation produced by the antimetabolite ([25], p. 76). A suggested explanation is that the excess metabolite slows up the reactions leading to its production in the tissue, by encouraging the reverse reactions, so that one of these reactions becomes growth-limiting. The tissue would then fail to increase its rate of growth in response to the increased velocity of the reactions in which the metabolite participates. The antimetabolite would not act in this way, but might even stimulate production of the metabolite by increasing the rate of its utilization.

If the theory described above provides the only explanation of the stimulatory effects of low concentrations of inhibitors, it follows that this phenomenon should be observed with competitive antagonists but not with inhibitors acting by other mechanisms. In fact, stimulation is observed with many different types of inhibitors [25]. However, this theory of stimulation is put forward, not as an alternative to the ones already proposed, but rather as a supplement. The growth processes of living organisms are too complex for there to be any great probability that a single explanation, such as this, is applicable in all instances.

A final implication of the foregoing theory of stimulation remains to be considered. If this theory is correct, it follows that for many enzyme reactions in living cells the "substrate-dissociation curve" (p. 585, 586) must be sigmoid instead of hyperbolic as predicted by the equation of Michaelis and Menten and the Langmuir adsorption isotherm. In other words, there is a "co-operative effect" in these enzyme reactions. Since equations (6) and (7) have been derived on the assumption that there is no co-operative effect, their ability to describe adequately *all* cases of competitive antagonism then becomes questionable. Until equations of the same form have been derived from others which make provision for the co-operative effect, equations (6) and (7) must be regarded as approximations. Krishnamurti [12] has proposed a new adsorption equation which does make such a provision, and this may serve as a basis for a new derivation of the relationship between the inhibition index and the substrate concentration. However, it should be noted that equation (6) adequately describes this relationship in the competition between carbon monoxide and oxygen for haemoglobin and in these reactions a co-operative effect is known to occur. It is probable, therefore, that equations (6) and (7), though approximations, are of general usefulness for the characterization of competitive antagonism.

SUMMARY

The theory of competitive antagonism has been reexamined and equations derived for characterizing it. It has been shown that the inhibition index[1] is not constant but is a linear function of the reciprocal of the substrate concentration.

In applying this relation to results of antimetabolite studies, it has been found that two types of graph result, those with a negative and those with a positive slope. It is suggested that these graphs distinguish two types of antagonist, the true competitive antagonist and the apparent competitive antagonist, the latter, in fact, undergoing the same reaction as the substrate with the formation of an inhibitory product.

With the help of equations derived above, the mode of action of sulphanilamide has been examined. The results suggest that sulphanilamide is metabolised in the same way as *p*-aminobenzoic acid with the formation of an inhibitory analogue of folic acid.

A theory to account for growth stimulation by low concentrations of inhibitors has been presented, and the means for testing it outlined. It is based on the postulate that the curve relating the concentration of enzyme-substrate complex to the concentration of enzyme is not hyperbolic but sigmoid, and it is developed by analogy with the known behaviour of carbon monoxide and oxygen towards haemoglobin.

I wish to thank Dr. M. Webb for the suggestion to base the theory on the laws of heterogeneous reactions, an idea from which all else developed, and Dr. C. S. Hanes for reading the first draft and pointing out the identity of the equations which had been derived on this basis with those derived from the laws of homogeneous reactions.

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[1] *Note added in proof.*—Perhaps the term "anti-inhibition index" would be better than "metabolic index" for the reciprocal of the inhibition index.

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ÉTUDE COMPARATIVE DES SÉRUMS DE POULE, DE COQ, DE POUSSIN, D'EMBRYON ET DU BLANC D'ŒUF

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Reçu le 30 Septembre 1955

L'étude des constituants protéiniques du sérum d'Embryon de Poulet au fur et à mesure du développement a fait l'objet de nombreux travaux. Nous l'avons reprise dans notre travail précédent [7] en utilisant une méthode nouvelle, l'immunoélectrophorèse [1, 2], qui nous a permis de déceler et de suivre, depuis le 5e jour d'incubation jusque chez le Poussin de 3 jours après l'éclosion la présence, souvent simultanée, de protéines provenant du Blanc d'Œuf ou du sérum de Poule.

Dans le but d'étendre et de compléter cette étude, nous avons tout d'abord établi une comparaison détaillée de ces deux milieux naturels extrêmes entre lesquels se poursuit l'évolution qualitative du sérum d'embryon, afin que les constituants spécifiques à chacun de ces milieux soient différenciés de constituants communs.

En deuxième lieu, nous avons recherché la présence éventuelle, parmi ces constituants initiaux et terminaux, de protéines embryonnaires proprement dites. Leur existence a été mentionnée [11] surtout dans les premiers jours du développement. Nous avons essayé de mettre en évidence ces constituants en utilisant leurs propriétés immunologiques.

Le dénombrement et l'identification des constituants protéiniques décelés dans les divers milieux étudiés ont été faits, comme dans le travail précédent, sur la base des propriétés électrophorétiques et immunochimiques. L'avantage des méthodes que nous avons utilisées dans ce travail est, comme nous l'avions déjà souligné précédemment, de permettre l'analyse d'un mélange complexe de protéines à l'état naturel.

MATERIEL

Antigènes

Sérum de Poule normale — SPN.—Nous avons utilisé le sérum de Poule non pondeuse, provenant d'un mélange de sang de deux Poules âgées d'un an environ, saignées à blanc. Sa concentration en protéines, après dialyse dans le tampon véronal, est de 2,6 % (par réfractométrie).

Sérum de Coq — SCoq.—Nous avons employé les échantillons provenant de deux Coqs : I et II. Le premier sérum a été employé dilué 2 fois avec de l'eau physiologique et le second non dilué. Leurs concentrations sont respectivement 1,96 % et 3,7 %. Le sérum de Coq II a été en partie fractionné par précipitation avec du sulfate d'ammonium : a) fraction précipitée entre 0 et 33 % de saturation : 0 33 %; b) fraction précipitée entre 35 et 50 % de saturation : 33–50 % et c) fraction restante : > 50 %.

Sérum de Poussin — SPoussin.—Le sang d'un poussin de 48 h a été prélevé à la carotide. Le sérum a été employé non dilué, sa concentration était de 2,6 %.

Sérums d'Embryons.—A partir des œufs de 8, 11, 14 et 17 jours d'incubation, nous avons prélevé le sang avec une seringue dans la veine vitelline, comme dans notre travail précédent [7], en prenant soin d'éviter toute contamination par d'autres liquides.

Nous n'avons pas prélevé de sang d'embryons plus jeunes que 8 jours à cause des difficultés de prélèvement sans contamination et surtout à cause des quantités infimes de sang pouvant être recueilli.

A partir de 24 œufs de 8 jours d'incubation, nous avons recueilli environ 1 ml de sang — *SE 8 j.*

Nous avons, d'autre part, prélevé les cœurs des embryons que nous avons sectionnés et lavés avec de l'eau physiologique. Ce liquide concentré par pervaporation, constitue : *Extrait 8 j.*

18 œufs de 11 jours nous ont fourni environ 3 ml de sérum — *SE 11 j;* 12 œufs de 14 jours environ 3,5 ml sérum — *SE 14 j;* et 12 œufs de 17 jours environ 4 ml sérum — *SE 17 j.*

La concentration approximative en protéines de ces sérums a été déterminée par réfractométrie, sauf pour celui de 8 j, semblant le moins concentré et qui était en quantité trop minime pour en prélever pour la mesure.

SE 11 j — 0,86 %, *SE* 14 j — 1,08 % et *SE* 17 j — 2,4 %.

Blanc d'œuf et ses constituants purifiés.—Nous avons employé le Blanc d'œuf homogénéisé au Waring blendor et centrifugé à 15.000 t/min., ce qui élimine la mucine. Le blanc a été ensuite dilué de moitié dans de l'eau physiologique — *BlO.*

Pour la comparaison de divers constituants du Blanc d'œuf avec ceux du sérum de Poule, nous avons utilisé, d'une part des préparations de protéines purifiées comme dans nos publications précédentes [3] : Ovalbumine *Ov*, Conalbumine ferrique — *CA* (don de M. R. Warner), Ovomucoïde — *OM*, Lysozyme — *Ly.* D'autre part, l'isolement de différentes Ovoglobulines α, β, et γ [3, 6] (très difficile au point de vue technique) n'étant pas encore achevé, nous avons employé une préparation complexe contenant une proportion élevée d'Ovoglobulines, précipitée au sulfate d'ammonium entre 0–50 % de saturation — *OGl 0–50 %.*

Tous les antigènes étaient conservés à l'état liquide à 0°, en présence de merthiolate de Na (1:10.000).

Immunsérums

Nous avons employé pour ce travail plusieurs antisérums soit contenant plusieurs anticorps différents, soit spécifiques pour un antigène purifié.

Les deux antisérums complexes principaux correspondent aux deux milieux naturels de référence; ce sont l'antisérum anti-Blanc d'œuf — *ABO* et l'antisérum anti-Sérum de Poule — *ASP.*

Ces deux sérums employés simultanément (voir mode opératoire) constituent en principe le système de référence complet, c'est-à-dire contenant théoriquement le nombre maximum d'anticorps spécifiques correspondant aux différentes protéines présentes dans les mélanges étudiés.

Cependant, nos précédentes études sur les Ovoglobulines [3, 6] ont montré que plusieurs de ces constituants du Blanc d'œuf ne sont pas facilement mis en évidence dans la réaction de précipitation Blanc d'œuf–antisérum homologue. Pour pouvoir identifier ces antigènes, nous employons toujours comme antisérum de référence complémentaire l'antisérum anti-Ovoglobulines — *AOGl.* Chaque mélange d'antigènes est d'abord analysé simultanément par rapport à ces trois antisérums afin d'identifier les constituants de base appartenant soit au système antigénique « Blanc d'œuf » soit au système « Sérum de Poule ». Pour déceler les constituants particuliers à l'état embryonnaire nous avons préparé des immunsérums anti-Sérums d'Embryons : Sérum anti-Sérum d'Embryons de 11 jours — *ASE 11 j;* de 14 jours — *ASE 14 j* et de 17 jours — *ASE 17 j.*

Pour vérifier la présence de certains constituants, nous employons des antisérums spécifiques : anti-Ovalbumine — *AOV* et anti-Conalbumine — *ACA.*

L'Ovomucoïde, le Lysozyme et les Ovoglobulines sont révélés par l'antisérum AOGl. Tous nos antisérums sont préparés en injectant aux lapins par voie intraveineuse des préparations d'antigènes adsorbés sur l'alumine.

Nos lapins ont reçu 2 mois d'injections, au lieu d'un, dans le but d'obtenir des sérums fortement précipitants.

En ce qui concerne les antigènes du Blanc d'œuf, les quantités de protéines utilisées pour l'immunisation ont été décrites dans nos publications précédentes [3, 6].

Pour les sérums de Poule et d'Embryons, nous avons employé 1 ml de sérum par lapin pour 1 mois d'immunisation.

Les immunsérums pauvres sont fractionnés à 33 % de saturation en sulfate d'ammonium dans le but de concentrer les anticorps.

MODE OPERATOIRE

Techniques électrophorétiques

Electrophorèse en veine liquide.—L'analyse électrophorétique selon Tiselius nous a servi comme base de comparaison des mobilités des constituants.

Les expériences sont faites dans les conditions décrites dans nos précédentes publications [3], en utilisant le tampon véronal sodique de pH 8,2 et $\mu = 0{,}1$.

Electrophorèse en milieu gélifié.—Nous employons la technique décrite par Grabar et Williams [1, 2] pour l'immunoélectrophorèse. La révélation des protéines déplacées est faite après séchage de la couche de gélose, fixation par l'alcool-acétique et coloration à l'Amido-Schwarz selon la technique décrite par Uriel et Scheidegger [15]. La pellicule colorée ainsi obtenue, une fois séchée, peut ensuite être décollée de son support en verre grâce à la présence de glycérine à 10 % dans le bain de fixage et photographiée par contact.

La quantité de produit employé est de 0,6 ml; de voltage aux bords de la plaque 80 v, soit un peu plus de 4 volts/cm, la durée d'électrophorèse 5 h.

Immunoélectrophorèse selon la technique de Grabar et Williams [1, 2]. –Le mode

opératoire est le même que pour l'électrophorèse en milieu gélifié mais la révélation est différente.

Les réservoirs à antigènes ont la forme ovoïde et contiennent 0,6 ml d'antigène mélangé à de la gélose deux fois plus concentrée que celle de la plaque.

La force ionique du tampon (véronal sodique pH 8,2) est de 0,025 dans la gélose et 0,05 dans les cuves. Le voltage est de 4 v/cm, la durée 5 h.

Nous employons l'antisérum mélangé à de la gélose, disposé en bandes longitudinales séparant les zones des antigènes.

Ayant à analyser chaque mélange d'antigènes par rapport à plusieurs antisérums différents (la plupart des liquides étudiés ont été révélés par 6 antisérums complexes) les réactifs ont été disposés de deux façons. Nous nous efforçons de faire chaque analyse sur une même plaque pour éviter les incertitudes d'identification dûes aux conditions opératoires variables, surtout en ce qui concerne le voltage. Pour effectuer le dénombrement complet de constituants d'un mélange, nous avons disposé sur une même plaque de 18 × 24, 5 réservoirs contenant la même substance. Chaque réservoir peut ainsi être encadré de deux bandes d'antisérums différents. Inversement, pour comparer divers liquides afin de voir s'ils contiennent un constituant donné, nous avons disposé jusqu'à 6 réservoirs contenant des antigènes différents; tous étant révélés par un même antisérum spécifique.

Enfin, pour certains antigènes, une vérification supplémentaire a été faite suivant le procédé que nous avons décrit dans le précédent travail [7], à savoir la dissolution de la ligne de précipité spécifique par un excès de l'antigène présumé déposé localement à proximité de cette ligne. On découpe pour cela dans le gel un petit réservoir, dans lequel on met 1 à 2 gouttes de préparation purifiée de cet antigène, en solution concentrée.

Précipitation spécifique en milieu gélifié

La technique immunochimique employée pour démontrer l'identité de deux antigènes est la précipitation par double diffusion selon Ouchterlony [12, 13).

Dans le présent travail nous employons deux dispositifs en boîte de Pétri, comme dans nos précédentes publications [8, 4, 3] — l'un à 4 réservoirs disposés en carré, qui permet la comparaison simultanée de deux antigènes et deux anticorps; l'autre à 6 réservoirs périphériques disposés aux angles d'un hexagone régulier qui permet la comparaison de plusieurs antigènes vis-à-vis d'un même antisérum contenu dans un réservoir central.

Les mélanges d'antigènes et les antisérums sont introduits non dilués dans les réservoirs. Les préparations de protéines antigéniques purifiées sont utilisées dans les dilutions correspondantes à l'équivalence du sérum.

L'évolution des traits de précipitation est assez longue dans nos conditions opératoires. Les lignes visibles le plus rapidement sont celles de la Sérumalbumine, de l'Ovalbumine et de la Conalbumine.

Pour voir les lignes correspondant aux globulines (la plus en retard est la α_1-Sérumglobuline) il faut attendre 8 à 10 jours et pour observer leur éventuelle jonction traduisant leur identité immunologique nous avons attendu 3 semaines. Pendant ce temps les boîtes de Pétri sont conservées dans une chambre à température constante (18°). On les photographie par contact, remplies d'eau physiologique pour rendre invisibles les défauts de surface de la gélose.

RÉSULTATS EXPERIMENTAUX

Pour chaque liquide étudié, les résultats sont indiqués dans l'ordre suivant: électrophorèse, immunoélectrophorèse, immunochimie. En effet, le classement de constituants principaux en groupes suivant leur mobilité est indiqué par le diagramme *d'électrophorèse en veine liquide*, ainsi que par coloration de taches après *l'électrophorèse en gélose*. Ces principaux groupes de constituants, aussi bien pour le Blanc d'œuf que pour le sérum de Poule, sont déjà connus depuis longtemps. Cependant, nous avons effectué l'analyse préalable dans l'appareil de Tiselius de chaque système étudié. Cette base d'appréciation des mobilités est nécessaire à cause de l'électroendosmose qui se produit lors de l'électrophorèse en gélose [1, 2].

Une différenciation plus fine des constituants est faite au moyen *d'immunoélectrophorèse*, surtout si on emploie plusieurs antisérums de référence contenant un nombre élevé d'anticorps différents.

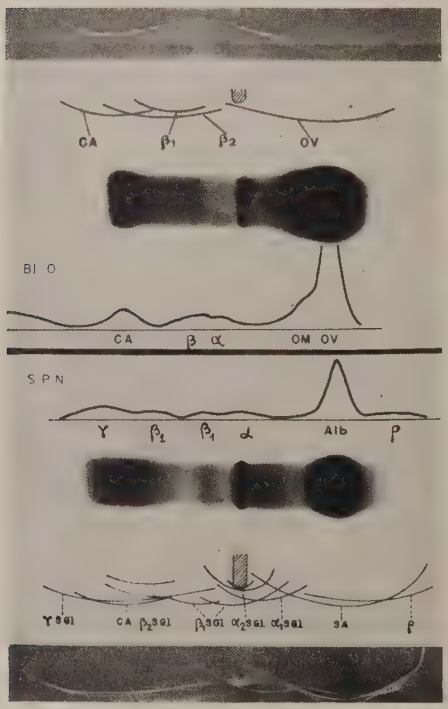

Fig. 1. Comparaison du Sérum de Poule et du Blanc d'œuf : diagramme d'électrophorèse selon Tiselius; électrophorèse dans la gélose colorée; immunoélectrophorèse révélée par l'antisérum homologue, photographie directe et schéma de toutes les lignes visibles durant l'évolution de la plaque.

La fig. 1 montre la comparaison de nos deux systèmes antigéniques de base : Blanc d'œuf et sérum de Poule. Les sommets du diagramme d'électrophorèse en veine liquide correspondent aux taches colorées d'électrophorèse en gélose et aux principales lignes d'immunoélectrophorèse avec l'antisérum homologue. (Cette dernière nous révèle cependant dans le sérum de Poule plus de constituants que l'électrophorèse : 11 au lieu de 6.) Cette comparaison nous montre que les principaux constituants des deux milieux ont des caractéristiques électrophorétiques respectives très voisines. Ainsi, dans le mélange contenant les protéines appartenant aux deux systèmes à la fois, l'analyse électrophorétique seule ne saurait être suffisante et l'emploi de l'immunoélectrophorèse est indispensable.

Enfin l'identification de constituants communs, obtenue par les *techniques immunochimiques,* est la dernière étape de l'analyse.

Les deux tableaux (I et II) reproduisent l'ensemble de tous nos résultats. Les constituants identifiés sont classés par ordre de mobilité électrophorétique en milieu gélifié, selon le sens du courant.

Constituants du sérum de Poule adulte. — Le diagramme *d'électrophorèse en veine liquide* (Fig. 1) montre 5 pics désignés Sérumalbumine, α_1, β_1, β_2 et γ Sérum globulines. En avant de la Sérumalbumine on devine l'existence d'un constituant plus rapide.

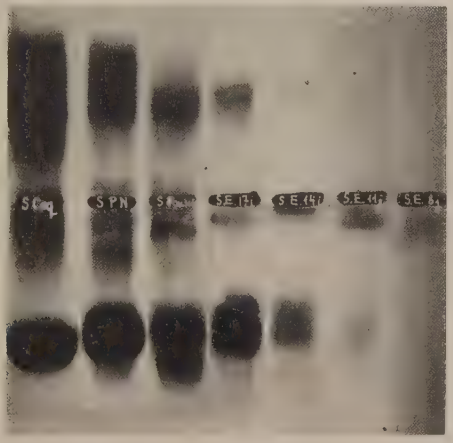

Fig. 2. Electrophorèse colorée dans la gélose. Sérum de Coq, Sérum de Poule, Sérum de Poussin de 2 jours, Sérums d'Embryons de 17, 14, 11 et 8 jours d'incubation.

L'électrophorèse en gélose (Fig. 1 et 2) montre une tache intense de Sérum albumine précédée de la trainée du constituant plus rapide. Une tache forte assez étalée de globuline α touche le réservoir d'origine dont l'emplacement reste fortement coloré, traduisant la présence de constituants non déplacés par

le courant dans nos conditions. De l'autre côté une tache de β-globuline se distingue avant une tache intense et très étalée sur l'emplacement des γ-globulines.

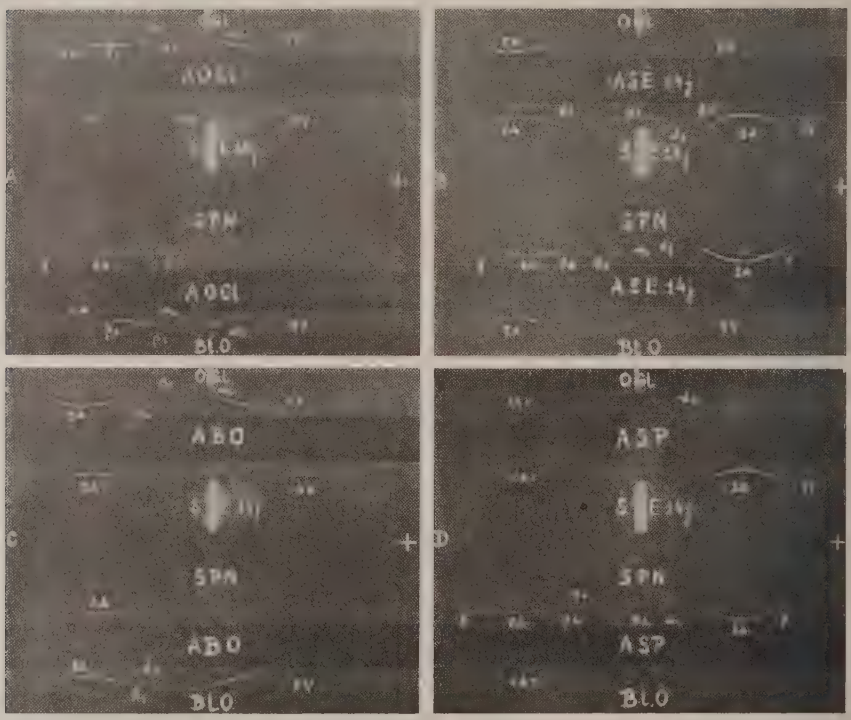

Fig. 3. Immunoélectrophorèses. Étude comparative de 3 milieux naturels : Blanc d'œuf, Sérum de Poule, Sérum d'Embryons de 14 jours et d'une préparation complexe : Ovoglobulines, révélés chaque fois avec un des 4 antisérums homologues : anti-Ovoglobulines (*A*), anti-Sérum d'Embryons de 14 jours (*B*), anti-Blanc d'œuf (*C*) et anti-Sérum de Poule (*D*).

L'immunoélectrophorèse avec l'antisérum homologue (Fig. 1 et 3*D*) montre un ensemble de lignes d'intensité inégale. Le plus près du pôle positif on aperçoit une ligne fine et nette correspondant au constituant le plus rapide, que nous avons nommé ϱ [7, 16]. Cette ligne se croise avec celle, très intense et large, de la Sérumalbumine, ce qui montre que les antigènes correspondants n'ont aucune parenté immunologique. On voit ensuite une ligne nette de α_1-Sérumglobuline et deux lignes de α_2. Ces dernières se trouvent tout près du réservoir d'origine et au même niveau que celui-ci, par rapport

à l'échelle des mobilités. Ces constituants semblent donc immobiles dans nos conditions expérimentales. Ce sont eux qui provoquent la coloration de l'emplacement du réservoir d'origine dans l'électrophorèse en gélose et ils ne diffusent presque pas. Leur immobilité peut être dûe, soit à leur charge électrique correspondant exactement aux conditions réalisées à cet endroit par la résultante du champ électrique, du coefficient de diffusion de la protéine et de l'électroendomose, soit à leur rétention par la gélose. Cette dernière peut être causée par une action spécifique chimique de la gélose sur la protéine.

De l'autre côté du réservoir se trouvent deux β_1-globulines, deux β_2-globulines, une ligne de mobilité intermédiaire entre β_2 et γ qui correspond à la Conalbumine et enfin une ligne très allongée et nette formée par la γ-Sérumglobuline. On peut à ce sujet rappeler la conclusion de Williams et Grabar [16] concernant la γ Sérumglobuline humaine et attribuant la forme allongée de la ligne à l'existence d'une famille de protéines de même spécificité et de mobilités différentes.

L'intensité relative des lignes et leur position (plus ou moins rapprochée de la zone d'anticorps) permettent de juger approximativement de l'importance quantitative du constituant donné. C'est ainsi que les trois anticorps présents en quantités les plus élevées dans le sérum anti-Sérum de Poule semblent être l'anti-Sérumalbumine, l'anti-γ-Sérumglobuline et l'anti-ϱ. On peut donc supposer que les trois protéines correspondantes sont, compte-tenu de la possibilité de différences dans leur antigénicité, les plus abondants constituants du sérum de Poule.

En ce qui concerne les α_2-globulines, il est difficile, par contre, d'apprécier leur quantité, car leurs lignes apparaissent très tardivement.

Les β_2-globulines et la Conalbumine sont parmi les constituants les moins abondants.

Avec l'antisérum anti-Blanc d'œuf (Fig. 3*C*) le sérum de Poule ne forme qu'une seule ligne en arc régulier. Cette ligne correspond à la Conalbumine. Elle est intense, car ABO contient beaucoup d'anticorps anti-Conalbumine. Elle se trouve, d'autre part, relativement plus loin du réservoir d'antisérum que la ligne analogue formée par le Blanc d'œuf. Ceci montre que le sérum de Poule contient moins de Conalbumine que le Blanc d'œuf. L'identification de la Conalbumine dans le sérum de Poule d'après l'emplacement de la ligne dans l'immunoélectrophorèse (remarquons qu'il y a une très légère différence de mobilité entre la Conalbumine sérique et celle du Blanc d'œuf) a été confirmée immunologiquement de plusieurs manières : (a) identité dans une épreuve selon Ouchterlony (Fig. 6*A*) entre un constituant du sérum

de Poule ou de Coq et la Conalbumine purifiée par rapport à ACA ou ASP (remarquons à ce propos que certains sérums ASP réagissent très faiblement avec la Conalbumine); (b) la ligne correspondant à la Conalbumine observée en immunoélectrophorèse de SPN avec ASP n'est plus visible lorsque l'immunsérum a été préalablement épuisé, soit par la Conalbumine purifiée, soit par le Blanc d'œuf; (c) l'épreuve inverse est aussi positive : le sérum ABO épuisé par le sérum de Poule ne réagit plus avec la Conalbumine purifiée; (d) la ligne de Conalbumine disparaît par dissolution sous l'effet de deux gouttes d'une solution de Conalbumine disposées à côté d'elle; (e) une démonstration plus directe enfin a été réalisée en ajoutant de la Conalbumine purifiée, respectivement aux deux fractions de sérum de Coq, l'une riche et l'autre très pauvre en ce constituant. A l'immunoélectrophorèse avec les sérums ACA et ASP, on observe dans le premier cas une intensification de la ligne de la Conalbumine par rapport au témoin et surtout son déplacement vers la zone d'anticorps. Dans le deuxième cas, la ligne formée est identique à celle formée par de la Conalbumine pure en quantité égale. Dans aucun cas n'apparaissait d'hétérogénéité dans l'aspect de la ligne. Ainsi l'apport de la Conalbumine agit uniquement dans le sens quantitatif.

Avec l'antisérum AOGl (Fig. 3*A*) le sérum de Poule réagit en formant trois lignes correspondant à : une γ-globuline, que nous identifions comme la γ-Sérumglobuline, la Conalbumine (ligne de même mobilité qu'avec ABO) et une β_1-globuline. Cette dernière, quoique de mobilité très voisine de celle des β-Ovoglobulines, est, le plus vraisemblablement, une Sérumglobuline.

Enfin les immunoélectrophorèses révélées par les immunsérums-anti-embryons (Fig. 3*B*) montrent les lignes de ϱ, Sérumalbumine, α_1, α_2, β_1 et β_2 Sérumglobulines, Conalbumine sérique et γ-Sérumglobuline (parfois deux lignes sont visibles).

L'analyse immunochimique selon Ouchterlony du sérum de Poule est assez compliquée du fait du nombre élevé d'antigènes et du manque de préparations de protéines isolées purifiées pouvant servir de référence. Nous avons fait plusieurs essais dans le but de vérifier la présence de certains antigènes et d'établir l'identité de quelques constituants avec du sérum de Coq ou des Embryons.

C'est ainsi que nous avons constaté l'absence d'Ovalbumine. En effet, aucun de nos lots successifs de sérum de Poule non pondeuse (chez la Poule pondeuse on en trouve des traces) [7, 10] ne réagissait avec le sérum spécifique anti-Ovalbumine, ni avec les anticorps anti-Ovalbumine de l'immunsérum ABO, lors de l'immunoélectrophorèse. De même l'Ovalbumine ne réagit pas avec ASP.

Par contre, l'identification de la Conalbumine est facile. Nous avons vu que la réaction est très nette dans le sens sérum de Poule–sérum anti-Conalbumine et elle est positive aussi, quoique moins marquée, dans le sens Conalbumine–ASP.

Dans nos conditions opératoires en boîte de Pétri, nous avons pu ainsi identifier quatre parmi les lignes formées dans la réaction homologue SPN–ASP (Fig. 6*C*). La ligne la plus rapprochée du réservoir d'anticorps, pas très intense, mais témoignant par sa position que la réaction se fait en excès d'antigène est celle de la Conalbumine. La ligne suivante, très rapprochée et beaucoup plus intense, de sorte qu'elle masque souvent la première, correspond à la Sérumalbumine. Ensuite, à peu près à mi-distance entre les réservoirs d'antigènes et d'anticorps, s'observe une ligne nette et fine, dont l'emplacement ne varie pas sensiblement avec le temps. Ceci indique l'équivalence du système précipitant. La position de la ligne suggère, de plus, que le poids moléculaire de l'antigène n'est pas de beaucoup inférieur à celui de l'anticorps. Nous attribuons cette ligne au constituant ϱ. Enfin le plus près du réservoir de l'antigène, on voit une petite ligne dont la faible intensité témoigne d'une faible quantité d'anticorps. Cette ligne est incurvée vers le réservoir d'antigène, ce qui signifie soit qu'il y a un excès d'anticorps, soit que l'antigène diffuse très faiblement. Nous attribuons cette ligne à la α_1-globuline.

Constituants du sérum de Coq.—La Figure 2 montre les différences qualitatives de *composition électrophorétique* existant entre les sérums de Poule et de Coq. Ce dernier en effet ne contient pas de protéine rapide ϱ (aucun de nos deux échantillons n'en contient[1]); il contient relativement plus de α_1-globulines et de γ-globulines que le sérum de Poule.

Ces observations sont confirmées par *l'immunoélectrophorèse*. En effet, la ligne du constituant ϱ manque dans le sérum de Coq, même avec des antisérums les plus riches en anticorps correspondants (Fig. 4). Par contre, on observe parfois deux lignes de γ-globulines. Les autres lignes sont les mêmes que pour le sérum de Poule. Aucune ligne n'apparaît à l'emplacement de l'Ovalbumine avec ABO.

Les immunoélectrophorèses des fractions du sérum de Coq montrent, par rapport à ASP que la fraction la moins soluble (0 à 33 % de sulfate d'ammonium) contient de la Sérumalbumine en forte proportion, des globulines α_1 et β, la Conalbumine et la γ-globuline. La fraction la plus soluble (non

[1] Nous signalons à ce propos une erreur dans la composition du tableau de notre publication précédente indiquant la présence de ϱ parmi les constituants du sérum de Coq. Dans les deux séries d'expériences nous nous sommes servis des mêmes lots de sérum.

précipitée à 50 % de sulfate d'ammonium) contient de la Sérumalbumine et de la Conalbumine en faible quantité. Seule la fraction intermédiaire contient des α_2-globulines. Elle contient en outre beaucoup de Sérumalbumine, des β_1 et β_2 globulines, de la Conalbumine et encore beaucoup de γ-globuline. Ces résultats sont analogues à ceux que nous avons observé lors du fractionnement du Blanc d'œuf [5] : la fraction la plus insoluble contient déjà une forte proportion d'Ovalbumine et c'est la fraction la plus rapprochée de la demi-saturation, considérée comme limite de précipitation des globulines, qui contient le plus de celles-ci.

Les *essais immunochimiques* par la technique d'Ouchterlony (Fig. 6 *A* et *D*) du sérum de Coq montrent, de même que pour le sérum de Poule, la présence de Conalbumine, l'absence d'Ovalbumine, et une forte proportion de Sérumalbumine.

Constituants du sérum de Poussin de 2 jours.—L'électrophorèse en gélose (Fig. 3) montre que le sérum de Poussin contient proportionnellement davantage de constituant ϱ que le sérum de Poule. La tache de l'Albumine est bien séparée de la tache de ϱ. On voit ensuite les α_1 et α_2 globulines, peu de β-globuline, la Conalbumine et très peu de γ-globuline. Toutes les taches sont moins intenses que dans le sérum de Poule, malgré la concentration en protéines égale.

Fig. 4. Comparaison du Sérum de Poule, de Poussin et de Coq.

L'immunoélectrophorèse (Fig. 4) révèle avec le sérum ASP les lignes suivantes : une ligne très intense correspondant au constituant ϱ, dont l'emplacement traduit un net excès d'antigène comparativement au système sérum de

Poule–même antisérum. La ligne suivante est celle de la Sérumalbumine. On voit ensuite deux α_1-globulines dont une plus rapide et n'existant pas dans le sérum de Poule, une α_2-globuline, une β_2-globuline, la Conalbumine et la γ-globuline, ligne nette mais dont l'emplacement traduit une teneur en cet antigène inférieure à celle du sérum d'adulte. Cette dernière ligne correspond de plus à une protéine légèrement plus rapide que la γ-Sérumglobuline de Poule. En effet, l'endroit où la ligne «γ» du sérum de Poule présente une courbure la plus accentuée correspond à une mobilité plus faible que celle de la courbure maximum de la «γ» chez le Poussin. Ceci indique que le constituant prépondérant des γ-globulines du sérum de Poule est plus lent que le constituant prépondérant des γ-globulines du sérum de Poussin.

Avec le sérum AOGl on voit des traces d'Ovalbumine, la β_1-globuline, la Conalbumine et la γ-Sérumglobuline de même caractéristiques qu'avec ASP. La différence entre la γ d'adulte et du Poussin n'est probablement qu'un léger décalage de mobilité sans modification de spécificité.

Avec les différents antisérums anti-Embryons, on voit successivement les lignes ϱ, Sérumalbumine, α_1 rapide, α_1 ordinaire, deux α_2, β_1, Conalbumine et γ-Sérumglobuline.

Constituants des sérums d'Embryons.—(*a*) *Embryons de 8 jours d'incubation.* —Du fait de la faible concentration en protéines du sérum *l'analyse électrophorétique en gélose* montre une seule tache au niveau des α_1-globulines (Fig. 2). On voit de plus une faible trainée qui traduit la présence de protéines rapides encore plus rapprochées du pôle positif que le constituant ϱ. L'emplacement du réservoir est intensément coloré.

L'immunoélectrophorèse avec ASP montre la présence de la protéine rapide ϱ en quantité importante, de deux α_1-globulines et de la Conalbumine (Fig. 5). Notons l'absence de ligne de Sérumalbumine. Nous avons déjà observé ce fait chez les embryons de moins de 8 jours dans notre travail précédent [7].

L'antisérum AOGl réagit seulement avec la Conalbumine (Fig. 5). Avec les sérums anti-Embryons, on voit ϱ, deux α_1-Sérumglobulines et la Conalbumine, comme avec ASP. En accord avec nos observations précédentes, la plupart des globulines ne sont pas apparentes chez les embryons au cours de la première semaine du développement.

Nous avons analysé aussi, pour le comparer au sérum, «l'extrait de 8 jours» (eau de lavage du cœur). L'image des lignes formées avec les mêmes antisérums est bien différente (Fig. 5) : ainsi avec ASP nous voyons une longue ligne presque droite, de mobilité difficile à déterminer (entre Albumine et ϱ); une ligne nette de α_1-globuline assez rapide et la Conalbumine. Avec AOGl, on voit une ligne nette d'Ovalbumine, une ligne de mobilité de α_2 qui

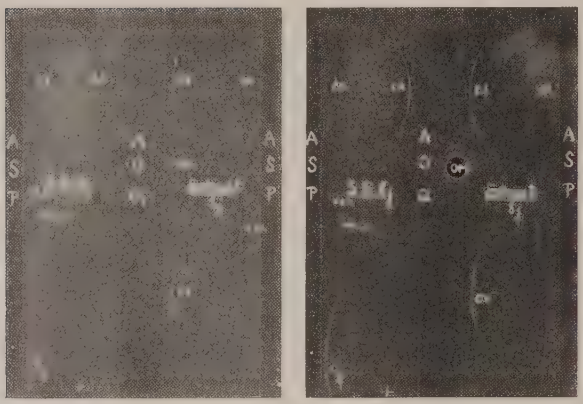

Fig. 5. Comparaison du Sérum d'Embryons de 8 jours et de l'eau de lavage du cœur (« Extrait 8 jours ») par rapport aux immunsérums anti-Sérum de Poule et anti-Ovoglobulines. Identification de la ligne correspondant à l'Ovomucoïde visible dans la réaction Extrait 8 jours–AOGl et qui disparaît en 12 h, dissoute par une solution d'Ovomucoïde purifié disposée dans le petit réservoir à côté.

est en réalité formée par l'Ovomucoïde (vérifié par dissolution à la goutte) et la ligne de la Conalbumine.

(b) *Embryons de 11 jours d'incubation.*—Ce sérum est aussi assez pauvre en protéines et les taches d'*électrophorèse en gélose* ne sont pas bien nettes (Fig. 2). On voit la trainée formée par ϱ plus rapide que dans le cas du sérum de Poule, l'Albumine, l'α_1-globuline et la Conalbumine. Le réservoir d'origine reste fortement coloré.

Les immunoélectrophorèses avec ASP montrent la présence du constituant ϱ rapide et de la Sérumalbumine. Avec l'immunsérum homologue ainsi qu'avec d'autres immunsérums anti-Sérum d'Embryons, on voit, en outre, une α_1-globuline rapide, une ligne très intense de la α_2-globuline et la Conalbumine.

L'immunsérum ASE 11 j est, des trois immunsérums anti-Sérum d'Embryon préparés, le plus pauvre en anticorps.

(c) *Embryons de 14 jours d'incubation.*—Ce sérum est plus riche en protéines que les deux précédents, ce qui nous a permis une analyse plus complète.

A l'électrophorèse (Fig. 2) on voit la même disposition des taches que pour les autres sérums d'Embryon. Ainsi, on observe d'abord une traînée correspondant à la protéine ϱ, ensuite une tache d'Albumine de forme carrée, ce qui correspond à la présence de deux taches voisines et confluentes, la α_1-globuline et la Conalbumine, L'emplacement d'origine est toujours très fortement coloré. On n'observe pas de tache nette de γ-globuline.

L'immunoélectrophorèse avec ASP (Fig. 3*D*) montre par rapport au sérum de Poule que la ligne de ϱ se trouve plus près du pôle positif, ce qui confirme l'emplacement avancé de la tache correspondante observée en

électrophorèse. Les deux lignes de ϱ, celle du sérum de Poule et celle du sérum d'Embryon (dans tous les sérums d'Embryons étudiés, il y a la ϱ rapide) ont toujours les mêmes positions respectives quel que soit l'immunsérum employé pour la révélation : anti-Sérum de Poule ou anti-Sérum d'Embryon. Il s'agit donc bien de constituants de mobilités différentes selon leur origine. Dans les deux cas, la jonction des deux lignes s'opère parfaitement (ce qui traduit l'identité immunologique) quand on dispose sur la plaque d'immunoélectrophorèse une bande d'antisérum transversale au niveau de la zone de migration du constituant ϱ.

La ligne de la Sérumalbumine est nette, par contre on ne voit pas bien ni les α, ni les β Sérumglobulines. On voit une ligne de Conalbumine assez faible, puisque le sérum ASP contient peu d'anticorps anti-Conalbumine, mais pas de ligne de γ-globuline, ce qui confirme les résultats d'électrophorèse. En ce qui concerne la γ-globuline, remarquons cependant que les anticorps correspondants existent dans le sérum ASE 14 j. En effet, avec le SPN, on observe une ligne de γ (Fig. 3*B*). Il est donc probable que l'antigène correspondant existe dans SE 14 j, mais vraisemblablement en trop faible quantité pour être observé dans la réaction homologue. Ce constituant est bien visible dans la réaction du SE 17 j et il se peut qu'il y en ait déjà des traces à partir de 14 j. L'explication de la ligne de γ-globuline de la réaction SPN–ASE 14 j n'est donc pas la même que pour la réaction SPN–AOGl.

Avec ABO on voit des lignes nettes d'Ovalbumine et de Conalbumine. (Fig. 3*C*).

Avec AOGl (Fig. 3*A*) nous voyons de plus sur l'emplacement entre α_2 et β_1 une ligne d'Ovomucoïde vérifiée par addition d'une goutte d'Ovomucoïde. On observe parfois (selon la distance entre le réservoir et la bande d'anticorps) une ligne doublant celle de la Conalbumine, mais presque droite. Cette ligne est une β-globuline car elle ne se dissout pas sous l'action d'un excès de Conalbumine et ne réagit pas avec le sérum anti-Conalbumine.

Avec l'antisérum homologue ASE 14 j, on voit, comme on pouvait le prévoir, le plus grand nombre de lignes (Fig. 3*B*).

Par ordre de mobilité, on observe successivement une ligne nette de ϱ rapide, celles de la Sérumalbumine, de l'Ovalbumine, de deux α_1-globulines, une ligne très intense de α_2-globuline, la β_2-globuline et la Conalbumine; la γ-Sérumglobuline n'apparaît pas.

La présence simultanée de deux Albumines, l'Ovalbumine et la Sérumalbumine, de mobilités bien voisines, explique la forme de la tache d'électrophorèse (Fig. 2).

Avec les autres sérums anti-Embryons, on voit les mêmes lignes.

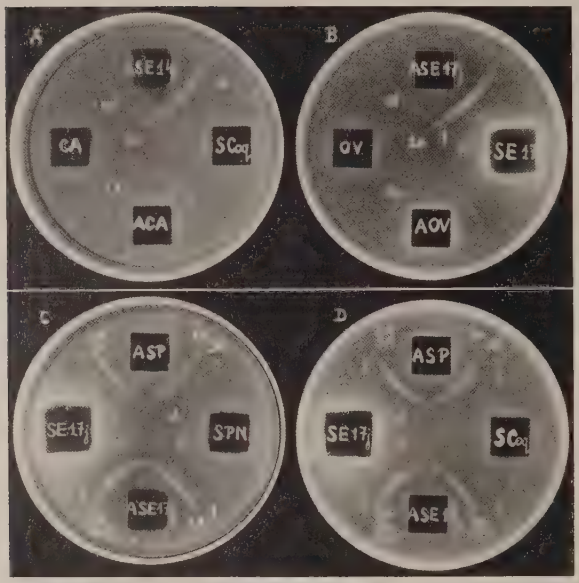

Fig. 6. Précipitation spécifique en milieu gélifié selon Ouchterlony.
A. Identification de la Conalbumine dans le Sérum de Coq et d'anticorps anti-Conalbumine dans l'immunsérum anti-Sérum d'Embryon de 14 jours. *B.* Identification de l'Ovalbumine dans le Sérum d'Embryons de 17 jours et d'anticorps anti-Ovalbumine dans l'immunsérum anti-Sérum d'Embryons de 17 jours. *C.* Comparaison du Sérum de Poule et du Sérum d'Embryons de 17 jours par rapport aux deux antisérums homologues. *D.* Comparaison du Sérum de Coq et de Sérum d'Embryons de 17 jours par rapport aux immunsérums anti-Sérum de Poule et anti-Sérum d'Embryons de 17 jours.

Des *tests immunochimiques* ont confirmé l'identification de l'Ovalbumine, de la Conalbumine et de certaines protéines sériques. Ils seront décrits plus en détail à propos du sérum d'Embryons de 17 jours; les deux séries, en effet, ont été faites de façon analogue, et la deuxième était plus complète, car nous disposions d'une quantité plus grande de sérum. Remarquons enfin que l'antisérum ASE 14 j est, parmi les trois sérums préparés, le plus riche en anticorps.

TABLEAU I

Tableaux des constituants du Blanc d'œuf, des Ovoglobulines, du Sérum de Poule, du Sérum de Coq, des Sérums d'Embryons de 8, 11 14 et 17 jours d'incubation et du Sérum de Poussin de 2 jours, d'après les résultats de l'électrophorèse en gélose et des immunoélectrophorèses avec des immunsérums anti-Blanc d'œuf, anti-Ovoglobulines, anti-Sérum de Poule, anti-Sérum d'Embryons de 11 jours, anti-Sérum d'Embryons de 14 jours, et anti-Sérum d'Embryons de 17 jours. Les constituants correspondant aux taches d'électrophorèse en gélose et aux lignes d'immunoélectrophorèse sont marqués dans l'ordre de leur migration dans la gélose : la ligne pointillée indique l'emplacement du réservoir d'origine; le pôle + se trouve vers le bas. La plupart des constituants révélés par l'immunoélectrophorèse sont définis par leur spécificité immunologique et sont alors désignés S, O ou E suivant qu'ils proviennent du Sérum de Poule, du Blanc d'œuf ou des Sérums d'Embryon.

Antigènes	Taches électrophorétiques	Anti-Sérum					
		ABO	$AOGl_3$	ASP	ASE 11 jours	ASE 14 jours	ASE 17 jours
BlO	CA	CA	CA	CA		CA	
			β_3 OGl				
	β Gl	β_2 OGl	β_2 OGl				
		β_1 OGl	β_1 OGl				
						α_2 Gl	
		α_1 OGl	α_1 OGl				
	OV	OV	OV			OV	
OGl 0–50 %	CA	CA	CA	CA		CA	
	β_3 Gl		β_3 OGl				
	β_2 Gl	β_2 OGl	β_2 OGl				
		β_1 OGl	β_1 OGl				
		α_2 OGl	α_2 OGl				
	α Gl	α_1 OGl	α_1 OGl	α_1 SGl			
	OV	OV	OV			OV	
SPN	γ Gl		2γ SGl	γ SGl		2γ SGl	2γ SGl
		CA	CA	CA	CA	CA	CA
	β_2 Gl			$2\beta_2$ SGl		β_2 SGl	β_2 SGl
	β_1 Gl		β_1 SGl	$2\beta_1$ SGl		β_1 SGl	β_1 SGl
				$2\alpha_2$ SGl		α_2 SGl	
	α Gl			α_1 SGl	α_1 Gl±	α_1 Gl±	α_1 Gl±
	A			SA	SA	SA	SA
	ϱ			ϱ	ϱ	ϱ	ϱ
SCoq	γ Gl			γ SGl		γ SGl	2γ SGl
	CA		CA	CA	CA	CA	CA
	β Gl		β_2 SGl	β_2 SGl		β_2 Gl	β_2 SGl
			β_1 SGl				
	α Gl			α_1 SGl±	α_1 Gl±	α_1 Gl±	α_1 SGl
	A			SA	SA	SA	SA

(d) *Embryons de 17 jours d'incubation.*—Ce sérum est le plus concentré des quatre, aussi les taches d'*électrophorèse en gélose* sont-elles les plus intenses. Cependant, la disposition reste la même. On voit ainsi une forte traînée de ϱ rapide, une tache intense mais encore assez carrée d'Albumine, la α_1, une traînée dans les β, la Conalbumine et un peu de γ-globuline (Fig. 2).

L'antisérum ASE 17 j est moins riche en anticorps que ASE 14 j. Ils sont, cependant, analogues au point de vue qualitatif.

Les immunoélectrophorèses avec différents antisérums donnent respectivement les mêmes résultats que pour le sérum précédent.

Tableau II

Antigènes	Taches électrophorétiques	Anti-Sérum					
		ABO	$AOGl_3$	ASP	ASE 11 jours	ASE 14 jours	ASE 17 jours
SE 8 jours	β Gl±		CA	CA		CA	CA
	α Gl			2 α_1 EGl	2 α_1 EGl	2 α_1 EGl	α_1 EGl±
	A±			ϱe	ϱe	ϱe	ϱe
SE 11 jours	β Gl		CA		CA	CA β_2 Gl	CA
	α Gl				α_2 EGl α_1 EGl	α_2 EGl α_1 EGl	α_2 EGl α_1 EGl
	A			SA	SA	SA	SA
	ϱ			ϱe	ϱe	ϱe	ϱe
SE 14 jours	β Gl	CA	CA OM	γ SGl± CA	CA	CA β_2 EGl	CA
	α Gl	OV	OV	α_1 EGl	α_2 EGl α_1 EGl	α_2 EGl 2 α_1 EGl OV	α_2 EGl 2 α_1 EGl ± OV
	A			SA	SA	SA	SA
	ϱ			ϱe	ϱe	ϱe	ϱe

Tableau II (*suite*)

Antigènes	Taches électrophorétiques	Anti-Sérum					
		ABO	$AOGl_3$	ASP	ASE 11 jours	ASE 14 jours	ASE 17 jours
SE 17 jours	γ Gl		γ SGl	γ SGl	γ SGl	γ SGl	γ SGl
			CA	CA	CA	CA	CA
	β Gl					β_2 Gl	β_2 Gl
			OM				
	----------		----------	----------	α_2 EGl	α_2 EGl	α_2 EGl
	α Gl				α_1 EGl	2 α_1 EGl	2 α_1 EGl±
			OV			OV	OV
	A			SA	SA	SA	SA
	ϱ			ϱe	ϱe	ϱe	ϱe
SP 2 jours	γ Gl		γ SGl	γ SGl		γ SGl	2 γ SGl
			CA	CA	CA	CA	CA
	β_2 Gl			β_2 SGl		β_2 Gl	β_2 Gl
	β_1 Gl		β_1 SGl	β_1 SGl			
	----------		----------	α_2 SGl	α_2 SGl	α_2 SGl	2 α_2 SGl
	α Gl			2 α_1 EGl	2 α_1 EGl	2 α_1 EGl	2 α_1 EGl
			OV±				
	A			SA	SA	SA	SA
	ϱ			ϱ	ϱ	ϱ	ϱ

Les tests immunochimiques ont été faits avec les deux antisérums ASE 14 j et ASE 17 j, après avoir vérifié que les deux réactions homologue et hétérologue sont qualitativement identiques.

Sur la Fig. 6*B* on voit que par rapport au sérum anti-Ovalbumine les lignes formées par l'Ovalbumine pure et par le SE 17 j se rejoignent parfaitement; ceci confirme les résultats de l'immunoélectrophorèse quant à la présence d'Ovalbumine dans SE 17 j.

Inversement, par rapport à l'antisérum ASE 17 j nous voyons que l'une des lignes formées par SE 17 j rejoint la ligne formée par l'Ovalbumine. Il y a donc de l'Ovalbumine dans le SE 17 j et de l'anti-Ovalbumine dans ASE 17 j.

D'autre part, des essais effectués d'après le même schéma que pour l'Ovalbumine ont permis d'identifier la Conalbumine dans les sérums d'Embryons et des anticorps anti-Conalbumine dans les antisérums anti-Embryons (Fig. 6*A*).

Ainsi, dans l'ensemble des lignes produites par la précipitation du système

SE 17 j–ASE 17 j (Fig. 6*B*), on identifie, en partant du bassin d'anticorps, d'abord la ligne de l'Ovalbumine; immédiatement derrière et souvent même superposée, se trouve une large et intense ligne correspondant à la Sérumalbumine. Cette ligne masque la ligne de la Conalbumine située presque au même emplacement. L'évolution de la plaque avec le temps montre bien après quelques jours la dissociation de deux lignes, mais au début les deux ou trois restent confondues. L'identification des autres lignes n'a pu être faite que partiellement.

L'étude (Fig. 6*C* et *D*) faite avec ASE 17 j, du SE 17 j, du SPN et du SCoq, permet, par ailleurs, d'identifier la ligne correspondant au constituant ϱ dans SE 17 j. Rappelons, en effet, que dans la réaction SPN–ASE 17 j les trois lignes les plus marquées correspondent à la Sérumalbumine, Conalbumine et ϱ. Les deux premières sont superposées. Dans le système SCoq–ASE 17 j il n'y a que les deux premiers des constituants précédents qui forment des lignes. La comparaison de deux réactions montre facilement que le constituant correspond à la ligne (marquée ϱ sur la photographie) qui rejoint une ligne identique dans la boîte où SE 17 j est comparé à SPN, alors que dans la boîte voisine (Fig. 6*D*) une ligne analogue fait défaut dans la réaction du Sérum de Coq.

La comparaison des sérums d'Embryons SE 14 j et SE 17 j par rapport aux antisérums correspondants montre que les deux systèmes précipitants contiennent les mêmes constituants : toutes les lignes formées se rejoignent entre elles, et leurs proportions seules sont différentes, les distances entre les lignes étant légèrement variables.

L'étude plus détaillée du *Blanc d'œuf* et des *Ovoglobulines* faisant partie d'une autre publication [6] nous résumons ici leur composition par rapport aux antisérums employés dans le présent travail (Fig. 3).

Le Blanc d'œuf contient d'après l'*immunoélectrophorèse* (Fig. 3*C*) avec l'antisérum homologue, de l'Ovalbumine, des Ovoglobulines β_1, β_2 et la Conalbumine. Avec AOGl, on voit de plus une ligne intense de la β_3-Ovoglobuline. Avec ASP une longue ligne faible de mobilité correspondant à la Conalbumine est seule visible. Avec ASE 14 j, nous voyons l'Ovalbumine, la α_2-globuline et la Conalbumine.

Les Ovoglobulines 0–50 % testées vis-à-vis de ABO montrent en plus des constituants identifiés dans le Blanc d'œuf, deux α_2-Ovoglobulines (dont le Lysozyme); avec AOGl (réaction homologue) nous avons de plus la β_3-Ovoglobuline. Avec ASP, il y a la même ligne de Conalbumine que pour le Blanc d'œuf et une α_1-globuline. Par rapport à ASE 14 j, enfin, on voit l'Ovalbumine et la Conalbumine.

DISCUSSION

Les résultats que nous venons d'exposer fournissent une liste de constituants qu'on peut classer dans l'ordre de leur migration électrophorétique dans la gélose et qui sont définis, pour la plupart, par leur spécificité immunologique.

De plus, ces résultats permettent d'étudier l'origine de ces constituants. Ceux-ci sont ainsi désignés, lorsque cette origine a pu être précisée, suivant qu'ils proviennent du sérum de l'animal adulte (Poule ou Coq), du sérum d'Embryons ou du Blanc d'œuf, par des lettres S, E ou O. (Eϱ, Sϱ, EAlb, SAlb, OvAlb (ou OV), α_1EGl, α_1SGl, α_1OGl, α_2SGl, α_2EGl, α_2OGl, OM, β_1Gl, β_1SGl, β_1OGl, β_2Gl, β_2OGl, β_3OGl, SCA, OCA, γSGl.)

En ce qui concerne le nombre de constituants que nous avons mis en évidence, il faut souligner que ce nombre ne représente que le minimum des antigènes éventuellement présents dans les milieux étudiés. En effet il est possible que certains de nos lapins n'aient pas formé d'anticorps contre un ou plusieurs de ces antigènes, ceci d'autant plus que nous avons utilisé pour l'immunisation des milieux contenant un nombre très élevé d'antigènes. C'est pour cette raison que nous avons toujours utilisé comme source d'anticorps des mélanges d'immunsérums différents. D'autre part le dénombrement des antigènes a été fait à l'aide de la précipitation spécifique en milieu gélifié et l'on sait que dans ces conditions le nombre de lignes de précipitation ne correspond qu'au minimum de constituants, deux constituants différents pouvant éventuellement précipiter au même endroit. Cette possibilité cependant, est beaucoup plus exceptionnelle en immunoélectrophorèse qu'avec la technique d'Ouchterlony. C'est pourquoi nous nous sommes servis de la première technique pour le dénombrement de nos antigènes. Il est possible enfin, qu'un constituant soit présent en quantité trop faible pour qu'on puisse observer un précipité, on sait en effet qu'il existe un seuil de concentration pour qu'une ligne visible se forme. L'existence d'un tel constituant peut cependant être mise en évidence d'une façon indirecte par la formation d'anticorps correspondants.

Nous envisageons maintenant les caractéristiques de chacun de ces constituants.

Constituant rapide ϱ.—Ce constituant a été décelé, aussi bien à l'électrophorèse qu'à l'immunoélectrophorèse du sérum de Poule. Le sérum ASP que nous avons utilisé dans ce travail réagissait bien avec ce constituant, mais parmi les lots précédemment utilisés plusieurs ne contenaient apparemment pas d'anticorps anti-ϱ. On pourrait expliquer ce fait par la faible anti-

génicité de ce constituant mais il semble que d'autres facteurs entrent en jeu, principalement la concentration. En effet, la teneur de notre sérum de Poule en constituant ϱ paraît proportionnellement plus faible que celle des sérums d'Embryons qui tous ont fourni des immunsérums particulièrement riches en anticorps anti-ϱ.

A ce propos Marshall et Deutsch ont signalé chez les embryons de 10 et 13 jours la présence dans le sérum, des constituants plus rapides que l'Albumine [9]. Ces auteurs ont constaté la disparition progressive de ces constituants dans les derniers jours d'incubation.

Le sérum de Poule utilisé dans ce travail provenait d'animaux jeunes; on peut donc supposer que pour cette raison il contient le ϱ, alors que chez les sujets plus âgés on en observerait moins au pas du tout. Nous ne pouvons dire si son absence dans les deux lots de sérum de Coq utilisés doit être imputée à la même cause.

D'autre part, nous avons vu que le constituant rapide n'a pas le même mobilité électrophorétique dans le sérum adulte et celui d'embryon. La spécificité immunologique est par contre identique. En effet, nous avons toujours observé une jonction parfaite de lignes provenant de la réaction de l'un ou l'autre de ces milieux et ceci par rapport aux deux antisérums correspondants. Ces faits peuvent être interprétés de deux façons. Si on se place du point de vue électrophorétique, il y a deux constituants rapides. Du point de vue immunologique c'est un seul constituant. Le fait que dans chacun de ces milieux nous ne trouvons qu'un seul constituant rapide à la fois plaide en faveur de la deuxième interprétation. En effet, dans le cas de deux constituants, on pourrait s'attendre à les trouver tous les deux présents, en proportions variables dans un des milieux d'origine.

Nous sommes donc vraisemblablement en présence de protéines ayant, suivant l'origine, un nombre variable de groupements polarisés responsables de la charge électrique mais possédant un même motif antigénique. Le fait que la spécificité immunologique soit stable semble indiquer que le «motif antigénique» est une structure complexe par rapport aux groupements polarisés et n'est pas affecté par leur variation quantitative.

Ces faits rappellent l'existence de deux constituants rapides dans le sérum humain [14]. En effet, dans les deux cas, les mobilités respectives des constituants rapides par rapport à celles de la Sérumalbumine humaine et de Poule sont très voisines. De plus, la ligne du constituant le plus rapide des deux est légèrement plus arquée et plus nette que celle du constituant plus lent.

Des réactions spécifiques colorées appropriées ont été effectuées pour évaluer la teneur en lipide des deux constituants rapides du sérum humain:

seul, le plus lent des deux fixe le Soudan noir. Ce fait expliquerait que n'ayant pas la même teneur en lipide, les deux constituants n'ont pas la même mobilité; il est probable de plus, que la diffusion soit gênée pour le constituant contenant des lipides et ainsi la ligne correspondante est plus floue et plus droite.

Cependant, dans ce cas les deux lignes sont visibles dans un même échantillon de sérum et elles se croisent. Ainsi, notre cas n'est pas vraiment analogue, car nos deux constituants rapides n'apparaissent jamais dans un même milieu et les deux lignes correspondantes se rejoignent parfaitement dans la technique d'Ouchterlony.

On peut d'autre part, rappeler ici le problème posé par la longue ligne des γ-Sérumglobulines dans le cas du sérum humain étudié avec l'antisérum de Cheval expliquée, comme l'ont suggéré Williams et Grabar [16], par l'existence de plusieurs protéines de mobilités différentes et de même spécificité. Notre cas particulier est cependant plus net puisqu'il n'existe que deux mobilités différentes. Il est possible enfin que les différences de mobilité que nous avons observées pour le constituant ϱ ne soient que deux valeurs extrêmes d'une variation progressive au cours du développement. Notons à l'encontre de cette hypothèse que le sérum du Poussin possède déjà un ϱ de même mobilité que le sérum de Poule.

Sérumalbumine. — L'apparition de la Sérumalbumine dans le sérum d'Embryons avait été signalée à partir du 5^e jour de l'incubation [11]. Dans notre travail précédent nous ne l'avons pas observé si tôt, la première identification de ce constituant remontant au 8^e jour. D'après nos résultats actuels le sérum d'Embryons de 8 jours ne contient pas de Sérumalbumine. Cette contradiction apparente s'explique par le décalage de date causé par une façon différente d'évaluer le développement de l'embryon. On peut, en effet, compter la durée du séjour à l'étuve, comme nous l'avons fait cette fois-ci, ou bien apprécier l'âge de l'embryon d'après son aspect morphologique, comme nous l'avions fait la première fois. La différence de temps peut facilement être de 24 h ou plus, et c'est suffisant pour que la Sérumalbumine ne soit pas encore visible à ce stade.

Chez les embryons plus âgés la Sérumalbumine est ensuite présente d'une façon stable et en concentration croissante.

Ovalbumine.—Nous n'avons jamais constaté la présence d'Ovalbumine dans les sérums de Poule non-pondeuse ou de Coq. Ces résultats sont en contradiction avec ceux de Marshall et Deutsch [10]. Ces auteurs ont en effet relevé dans ces milieux de faibles quantités d'Ovalbumine, mais cependant mesurables par les méthodes immunochimiques quantitatives.

En ce qui concerne la présence d'Ovalbumine dans les sérums d'embryons, nous l'avons décelée chez les embryons de 14 et 17 jours. Dans notre travail précédent nous l'avions observée régulièrement dans le sérum d'Embryons de tous les âges. Il est donc probable d'admettre qu'elle existe aussi dans nos échantillons de 8 et 11 jours mais, ne l'ayant pas mise en évidence, à cause vraisemblablement de la très faible concentration en protéines totales de ces deux sérums, nous ne l'avons pas mentionné parmi les résultats.

Ovomucoïde.—Dans notre travail précédent, nous avons signalé tantôt la présence, tantôt l'absence d'Ovomucoïde dans le sérum d'Embryons au cours du développement [7]. La cause de ces apparitions et disparitions répétées n'a pas été suffisamment éclaircie. Nous les avions attribuées à un entraînement par les globules rouges du sang. Des expériences ont été faites depuis pour confirmer cette hypothèse mais nous n'avons pas eu de résultats très probants. D'autre part, l'expérience de l'immunoélectrophorèse comparative du sérum d'embryons de 8 jours et de l'eau de lavage des cœurs nous offre une autre possibilité d'explication. En effet, dans notre travail précédent, dans beaucoup de cas, afin d'augmenter la quantité de matériel, nous avions ajouté au sang pur, recueilli dans la veine vitelline, de l'eau de lavage des cœurs d'embryons. Nous savons maintenant que cette préparation contient de l'Ovomucoïde même quand le sérum pur n'en contient pas. Remarquons que la présence d'Ovomucoïde dans les sérums d'Embryons de 14 et 17 jours est réelle, le sang recueilli pour ces deux séries d'œufs incubés provenant uniquement de la veine vitelline.

Dans les antisérums ASE 14 et 17 j, nous n'avons cependant pas constaté la présence d'anticorps anti-Ovomucoïde. Nous attribuons ce fait à la faible antigénicité connue de ce constituant d'une part, et à sa faible concentration dans les sérums d'embryons d'autre part. En effet, la ligne du SE 14 j avec AOGl se trouve assez loin de la zone d'anticorps, or nous savons que l'Ovomucoïde est un antigène diffusant rapidement [6].

En ce qui concerne le Blanc d'œuf et les Ovoglobulines, nous n'avons pas observé de ligne d'Ovomucoïde. Il est présent en petites quantités dans ces milieux et la ligne n'est visible que dans la réaction de certaines préparations complexes ou d'Ovomucoïde purifié, avec le sérum AOGl [6].

Conalbumine et γ-Sérumglobuline.—A cause de la discussion existant dans la littérature sur les rapports entre ces deux protéines nous les discutons ensemble dans un même paragraphe. Nous avons déjà signalé la présence de la Conalbumine dans le sérum de Poule lors de notre travail précédent et comparé nos conclusions à celles de Marshall et Deutsch [10]. Rappelons que ces auteurs ont isolé la Conalbumine du sérum de Poule et l'ont comparée à

celle du Blanc d'œuf. Les deux ont été trouvées identiques au point de vue immunochimique. Nos résultats confirment pleinement l'individualité de la Conalbumine dans les sérums de Poule et de Coq. Les anticorps anti-Conalbumine sont d'autre part présents dans l'antisérum ASP. Les expériences d'épuisements croisés : ABO épuisé par SPN et ASP épuisé par BlO montrent, par ailleurs, l'identité immunochimique de l'Ovo-Conalbumine et de la Séro-Conalbumine. Cette protéine est différente de la γ-Sérumglobuline bien que souvent on les trouve ensemble dans les préparations fractionnées.

L'aspect irrégulier de la ligne formée lors de l'immunoélectrophorèse entre le Blanc d'œuf et l'anti-Sérum de Poule (Fig. 3*D*) mérite d'être discuté. Cette ligne, de même que celle des Ovoglobulines ou du sérum d'Embryons de 14 jours ou de la Conalbumine purifiée est allongée et ressemble davantage à celle de la γ-globuline. Cependant, à notre avis, on ne doit pas imputer cette ligne à la présence de la γ-globuline dans le Blanc d'œuf pour des raisons quantitatives. En effet, l'antisérum ASP contient beaucoup d'anticorps anti-γ-globuline et peu d'anti-Conalbumine, puisque la ligne correspondant au premier antigène dans la réaction SPN–ASP est intense, alors que celle de la Conalbumine est faible. Dans les réactions du Blanc d'œuf, des Ovoglobulines et du sérum d'Embryons avec ASP, les lignes formées sont toujours très faibles et rapprochées du bassin d'anticorps. Or, si ces lignes étaient dûes à la γ-globuline, elles devraient être intenses (quantité d'anticorps élevée). En supposant, d'autre part, la quantité d'antigène faible, par rapport à celle d'anticorps, la ligne se formerait loin du bassin d'anticorps. Ces deux suppositions sont infirmées par les résultats. Par contre, en faveur de l'identification de ces lignes comme correspondant à la Conalbumine viennent les faits que les systèmes antigéniques en cause contiennent tous une proportion élevée de Conalbumine, cependant que l'antisérum utilisé ne contient pas assez d'anticorps spécifique à cette protéine pour produire une ligne intense.

D'autre part, bien que la présence de γ-globuline dans le Blanc d'œuf ne soit pas, à priori, impossible, puisque l'on sait que certains anticorps sont transmis par l'œuf, nous ne croyons pas que le fait d'observer une ligne de γ-globuline entre SPN et AOGl soit un argument suffisant pour admettre la présence de la γ-Sérumglobuline dans le Blanc d'œuf. En effet, ni le Blanc d'œuf, ni les Ovoglobulines (pourtant concentrées en globulines par rapport au Blanc d'œuf) ni le sérum d'Embryons de 14 jours (qui contient vraisemblablement des traces de γ-globuline) ne forment cette ligne avec AOGl.

Ce fait curieux peut être expliqué, soit par une parenté immunologique

entre certaines des globulines du sérum de Poule et du Blanc d'œuf, soit par un affaiblissement de la spécificité «anti-protéine du Blanc d'œuf» de l'immunsérum AOGl comparativement à ABO, ne produisant pas, lui, cette ligne. La première hypothèse se traduirait vraisemblablement par l'apparition plus fréquente de lignes de globulines, lors des réactions croisées SPN–ABO et BlO–ASP.

Nous pensons plutôt que l'antisérum AOGl utilisé dans ce travail, du fait qu'il provenait d'animaux hyperimmunisés, avait une spécificité moins stricte et réagissait ainsi avec certaines globulines quelle que soit leur provenance, Blanc d'œuf ou sérum de Poule.

Un fait analogue, par ailleurs, est la formation d'une ligne de β_1-globuline lors de la même réaction. Cette ligne correspond par son aspect et son emplacement à une quantité élevée d'anticorps et une quantité relativement faible d'antigène. Or, d'autre part, dans la réaction du sérum AOGl avec son antigène homologue les Ovoglobulines, nous n'observons aucune ligne intense de même mobilité. Nous pensons donc qu'une fois de plus des anticorps anti-Ovoglobulines du sérum AOGl ont réagi avec une β-Sérum-globuline à cause de ses caractères «globulines». Remarquons, à l'appui de notre hypothèse, que dans les deux cas γ et β, le sérum ABO (réagissant moins fortement avec les globulines que AOGl) ne donne pas de lignes correspondantes.

En ce qui concerne la Conalbumine, nous avons observé de légères différences de mobilité entre la Conalbumine du sérum (plus rapide) et celle du Blanc d'œuf. Ces différences quoique moins nettes que celles rapportées au sujet de ϱ, nous semblent de même nature car la spécificité immunologique «Conalbumine» est unique.

Enfin, du point de vue général, il faut souligner l'analogie entre les caractéristiques physiques et chimiques de la Conalbumine et de la Sidérophiline du sérum humain. Toutes les deux sont des protéines capables de fixer le fer; leurs poids moléculaires sont presque identiques : 87.000 et 90.000; leur migration électrophorétique est la même : toutes les deux en effet sont des β-globulines assez lentes. Il se peut donc qu'il s'agisse d'un constituant identique ou du moins très voisin. Remarquons toutefois que la capacité totale du sérum de Poule de fixer le fer est supérieure à celle de la Conalbumine présente, seule [10].

Constituants embryonnaires.—Nous avons vu que les sérums d'Embryons contiennent, en plus des protéines du sérum de Poule et du Blanc d'œuf, des constituants que l'on n'observe que chez eux.

En dehors du constituant ϱ dont la mobilité électrophorétique seule est

différent, de celle de son analogue sérique, ce sont surtout des globulines qui attirent notre attention sur les plaques d'immunoélectrophorèse.

Les α_1-globulines embryonnaires sont généralement plus rapides que les α_1 sériques. Leurs lignes se forment avec tous les sérums d'Embryons et le sérum de Poussin en présence d'immunsérums anti-sérums d'Embryons et aussi, mais plus faiblement, avec ASP. Il semble donc qu'on se trouve, encore une fois, en présence d'une modification de structure d'un constituant concernant plutôt la charge de la molécule que son motif antigénique. Cependant, ici, l'intensité de la ligne, contrairement à ce qui se passe pour ϱ, est différente suivant qu'on considère la réaction homologue ou hétérologue. Cette intensité de la réaction homologue est quand même trop faible pour permettre une étude plus détaillée par la technique d'Ouchterlony et l'apparition tardive de lignes augmente encore cette difficulté. Nous ne pouvons donc dire encore s'il s'agit pour les α_1 embryonnaires et sériques d'une même spécificité immunologique, la quantité d'anticorps dans ASP étant plus faible que dans ASE, ou bien d'une modification de la spécificité partielle ou totale.

En ce qui concerne la α_2-globuline, la différence d'aspect des lignes est assez frappante. Outre un faible décalage de mobilité entre la α_2 embryonnaire et sérique, la première forme une ligne très intense dans les réactions entre les sérums d'Embryons de 11, 14 et 17 jours, et les immunsérums homologues. Il n'y a pas de formation de ligne analogue dans la réaction sérum d'embryons–ASP. Il s'agit donc très nettement d'un constituant différent purement embryonnaire. L'intensité de la ligne prouve une quantité élevée d'anticorps et sur certaines plaques cette ligne, large et diffuse, se déplace loin vers le bassin d'anticorps témoignant de la présence d'un excès d'antigène. C'est donc un constituant spécifiquement embryonnaire et quantitativement important.

Un autre «constituant embryonnaire» est l'Ovomucoïde qui est remplacé, dès l'éclosion par une β_1-Sérumglobuline. Nous avons déjà rapporté ce fait [7]. A ce propos, nous devons souligner des différences de nomenclature de constituants entre ce travail et le précédent. Par suite des conditions opératoires modifiées (force ionique du tampon et voltage) nous n'avons pas la même électroendosmose, d'où il s'ensuit un décalage dans l'emplacement des lignes.

Enfin, des constituants de mobilité voisine de celle des Albumines, sont aussi visibles sur nos plaques dans les sérums d'Embryons de 14 et 17 jours. Ces lignes sont faibles et généralement droites. Nous ne les avons pas identifiées jusqu'à présent.

Les Globulines.—Le problème de la spécificité des globulines n'est pas entièrement résolu. Ainsi l'identification des α_1-Ovoglobulines et des β_1 et β_2 Sérum- et Ovoglobulines n'est pas encore tranchée. Les lignes correspondantes en effet sont toutes assez faibles, leurs mobilités respectives sont les mêmes et il se peut qu'avec les moyens immunologiques on ne puisse résoudre ce problème. L'investigation immunologique se base effectivement sur la spécificité de la réaction antigène–anticorps. Dans les cas où cette spécificité, comme nous l'avons vu pour certaines globulines, n'est plus tout à fait stricte, nous perdons la possibilité de vérifier nos résultats. Cette diminution de la spécificité que nous observons pourrait être, en accord avec l'opinion généralement admise, due à l'hyperimmunisation des lapins qui nous ont fourni l'immunsérum AOGl. Cependant, il semble bien à la lumière de nos résultats, que cette diminution de la spécificité ne concerne que quelques constituants que nous ne pouvons pas déceler avec d'autres antisérums. Ne pouvant pas étudier ces constituants avec les antisérums qui ne forment pas de lignes avec eux, nous sommes obligés d'utiliser le sérum AOGl. Il ne nous semble donc pas que l'hyperimmunisation soit vraiment en cause dans notre phénomène de diminution de spécificité.

Une difficulté de même ordre s'est déjà posée pour la γ-Sérumglobuline, mais dans ce cas particulier les caractéristiques quantitatives des réactions nous ont permis l'identification de cette protéine. Pour les α et β globulines sériques ou du Blanc d'œuf, les lignes formées sont généralement faibles. Elles sont un peu plus nettes lorsque l'antigène est le Blanc d'œuf, mais la différence n'est pas suffisante pour permettre avec sécurité une identification. Ces lignes disparaissent aussi bien lorsqu'on épuise le ASP avec du Blanc d'œuf et le ABO avec du sérum de Poule. Ces faits plaident en faveur d'une spécificité commune mais en absence d'autres arguments, nous ne pouvons pas l'affirmer.

CONCLUSIONS

Les résultats que nous venons d'exposer montrent la composition antigénique des différents milieux étudiés, sous réserve que le nombre des constituants énumérés n'est que le nombre minimum de constituants décelables dans nos conditions opératoires et avec les immunsérums que nous avions à notre disposition.

Le Sérum de Poule non-pondeuse contient le constituant ϱ, la Sérumalbumine, des α_1 et α_2 Sérumglobulines, des β_1 et β_2 Sérumglobulines, la Conalbumine et la γ-Sérumglobuline.

La Conalbumine est un constituant immunologiquement et électrophorétiquement différent de la γ-Sérumglobuline.

Le Sérum de Poule Pondeuse contient, en plus des constituants du sérum de Poule non-pondeuse, des traces d'Ovalbumine.

Le Sérum de Coq contient la Sérumalbumine, une α_1-Sérumglobuline, des β_1 et β_2 Sérumglobulines, la Conalbumine et la γ-Sérumglobuline.

Le Sérum de Poussin de 2 jours contient le constituant ϱ, la Sérumalbumine, des traces d'Ovalbumine, les α_1-globulines embryonnaires, des α_2-Sérumglobulines, des β_1 et β_2-Sérumglobulines, la Conalbumine et la γ-Sérumglobuline.

Le Sérum d'Embryon de 8 jours contient le constituant ϱ rapide (ϱ embryonnaire), des α_1-globulines embryonnaires et la Conalbumine. La présence de l'Ovalbumine a en outre, été démontrée précédemment [7].

Le Sérum d'Embryon de 11 jours contient le constituant ϱ embryonnaire, la Sérumalbumine, de l'Ovalbumine [7], des α_1-globulines embryonnaires, la α_2-globuline embryonnaire, une β_2-globuline et la Conalbumine.

Le Sérum d'Embryon de 14 jours contient le constituant ϱ embryonnaire, la Sérumalbumine, l'Ovalbumine, des a_1-globulines embryonnaires, la α_2-globuline embryonnaire, une β_2-globuline, la Conalbumine, et vraisemblablement, des traces de γ-Sérumglobuline.

Le Sérum d'Embryon de 17 jours contient le constituant ϱ embryonnaire, la Sérumalbumine, l'Ovalbumine, des α_1-globulines embryonnaires, la α_2-globuline embryonnaire, l'Ovomucoïde, une β_2-globuline, la Conalbumine, la γ-Sérumglobuline.

SUMMARY

The constituents of serum of hen, rooster, newly hatched chick were compared to those of the chick embryos of 8, 11, 14 and 17 days and to the constituents of hen egg white by immunochemical precipitation by double diffusion in agar, immunoelectrophoresis and zone electrophoresis in agar gel.

The α globulins of adult chick serum were different from those of embryo serum. Both adult and embryo serum contain a constituent moving more rapidly in electrophoresis than Serumalbumin (named ϱ) but it has a greater mobility in embryo than in adult hen, however its immunological specificity is the same. Some constituents of egg white are present only in embryo serum (i.e. Ovalbumin and Ovomucoïd) but all the sera contain Conalbumin, immunologically the same as in egg white.

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THE EFFECT OF WATER UPON GAMETES, UPON MATURATION, AND UPON FERTILIZATION AND CLEAVAGE[1]

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WATER is so basic a constituent of protoplasm that it is much taken for granted, and the effect of water itself upon basic phenomena has not received a great deal of attention experimentally. Yet relative amount of water, even if only by determining the distance between reacting particles, may well be a controlling factor in the functioning of protoplasm.

The ova of many marine invertebrates are favorable material for the study of the effects of water, since additional amounts may easily be introduced by dilution of the external medium. Such is not the case with the eggs of vertebrates, where the cells are insulated against water exchange [4, 11]. In this paper it will be shown that the gametes of the surf-clam, *Spisula* (also known as *Mactra*) *solidissima*, are highly tolerant to dilution, and that the zygotes are less tolerant, as evidenced in the inhibition of cleavage. Also, that one stage of maturation is highly sensitive.

MATERIAL AND METHODS

The essential characteristics and behavior of the ova of *Spisula* (then referred to as *Mactra*) are described in a previous paper [12]. The present report is based upon certain experiments carried on at the Marine Biological Laboratory at Woods Hole, Mass. in 1952–55, during the month of July of each year.

The fact that the *Spisula* egg is shed in the germinal vesicle stage makes possible direct observation of the osmotic response of the egg as a whole and also of the nucleus. Quantitative studies were performed by measurement of the diameter of the egg and of the diameter of the germinal vesicle. From this data changes in the volume and in the surface area of the egg and of the nucleus were computed. By subtracting nuclear volume from egg volume changes in the cytoplasm were obtained. Measurements were made upon individual egg cells with a 20× ocular and a 60× objective. With this combination one unit on the ocular scale covered a span of 1.82 microns of field.

It is of interest that the diameter of the egg in sea water, as currently measured,

[1] A portion of the expenses of this work was reimbursed from a research fund administered by the President of the College.

was 54.7 microns, based on 100 or more individual measurements. That of the germinal vesicle was 31 microns. As reported in the 1941 paper, the egg was 53 microns and the germinal vesicle 31. Allen [1] found a diameter of 56 microns. His measurements of the germinal vesicle, and of the nucleolus—11 microns agree with mine of 1941 and currently. The difference in egg diameter, although slight, is probably not due to experimental error. Slight changes in the salinity of the water could account for it. Also, temperature variations— which cause expansion of the water and, therefore, relative variation in the concentration of solutes—might be involved. In the Woods Hole area there have been significant changes in the temperature of the sea water. For the work reported in the 1941 paper a sea water temperature of 17° C prevailed at the beginning of July, and a maximum of 24° C was not reached until the end of August. During recent years the temperature varied from 21° C early in July to 23.5° C at the end of the same month.

In present studies, in order to obtain eggs, gonads were excised and sectioned in sea water essentially in the manner described by Allen. Stripping the gametes manually from the gonad, the method previously employed, proved ineffective when applied to the larger clams obtained from Barnstable beds; and the latter were used in most of the experiments now reported.

In procedure two or three drops of concentrated egg suspensions, about 0.1 cc, were added to 20 cc of solution in a covered 25 cc Stender dish. Except for experiments of short duration, the dishes were placed on a sea water table for temperature control. Dilutions, from undiluted sea water down to a mixture of 40 per cent sea water and 60 per cent distilled water, were made in 10 per cent steps.

It might be noted that *Spisula* eggs in distilled water become cytolyzed within two minutes. In 5 per cent sea water they persist for about four minutes, and in 10 per cent sea water for about 15 minutes. In 40 per cent sea water only a very occasional egg is destroyed after two or three hours of exposure. Eggs from 40 per cent sea water regained their original size in natural sea water and were fertilizable. Seventy per cent sea water proved optimal for survival. Eggs in this dilution outlasted those in other dilutions and also the eggs in natural sea water.

Osmotic equilibrium of this type of material is very quickly attained. Lucke and Ricca [10] found that oyster eggs, in the same range of dilution as here employed, completed their response within five minutes. The *Spisula* egg, when placed in 40 per cent sea water, swelled from its initial diameter of 54.7 microns to 67.4 microns within two minutes and, when returned to natural sea water, water rushed out just as quickly. Under the latter stress the stiffer outer covering of the egg, the vitelline membrane, became buckled, but the cytoplasm within its plasma membrane was rounded and reduced to its original volume. Zygotes reach the same size in 40 per cent sea water as the unfertilized egg, and return to the same volume—with a crenulated vitelline membrane—when restored to natural sea water.

In the range of dilution employed the question of possible effect of pH change might be raised. Several tests showed that there is no significant drop in pH until a dilution containing less than 20 per cent sea water. On the other hand, there appeared to be a slight initial rise in pH with dilution. The average of three determinations made with a Beckman meter and glass electrode showed a pH of 8.14 for sea water. This rose to a maximum of 8.21 in 60 per cent sea water, fell to 8.18 in 40 per cent and finally dropped to 7.8 in 10 per cent sea water.

The Effect of Water upon Spisula Ova

The data of this section is concerned with unfertilized eggs, but it should be noted here that ova from the various dilutions could be returned to natural sea water after an hour or two and they were then fertilizable. There was no apparent difference in cleavage and they would develop into swimming larvae within six hours.

Table I shows the change in size of the egg, computed from the measured increase in diameter as listed. It is evident that the egg is capable of withstanding an inflow of water which almost doubles its volume, while the surface area is increased by about 50 per cent. These stresses cause no lasting injury. From the fourth column of Table I it is apparent that the percentage of increase in volume is not constant. Except for the step from 90 to 80 per cent sea water, there is a gradually accelerating effect. The slight retardation of swelling, from 90 to 80 per cent, occurs when the egg diameter has increased by about two microns. This may, perhaps, be attributed to the compound nature of the egg surface. The force of initial swelling is probably opposed largely by the plasma membrane alone. After the slack between the plasma membrane and the outer vitelline membrane is taken up, with an increase in egg diameter of about two microns, presumably both act together as a single force. The unusually large increment per cent between 50 and 40 per cent sea water may be related to the impending cytolysis of the cell.

Water from the medium dilutes the cytoplasm, and this dilution is osmotically transmitted to the germinal vesicle. It is evident that in the same range

TABLE I

Response of the egg to dilution of sea water.

Sea water percentage	Diameter of egg in microns	Egg volume	Increment per cent	Egg surface	Increment per cent
100	54.7	86,000		9,410	
90	56.4	94,200	9.5	10,000	5.9
80	58.0	102,300	8.6	10,570	5.7
70	60.1	113,500	10.9	11,370	7.6
60	62.2	126,000	11.0	12,140	6.8
50	64.4	140,000	11.1	13.000	7.1
40	67.4	160,500	14.6	14,260	9.7
Total increment		74,500	87.0	4,850	51.5

of dilution the volume of the germinal vesicle enlarges a great deal more, percentagewise, than does the egg as a whole, as shown in Table II. The vesicle shows an increase to more than twice its original volume, and its surface enlarges 76 per cent. This greater reaction of the germinal vesicle to dilution of the sea water may be significant with regard both to the survival of the unfertilized egg and to the findings on maturation and cleavage, to be reported below.

Since the water which enters the germinal vesicle is separated osmotically by the nuclear membrane from the water which dilutes the cytoplasm,

TABLE II

Osmotic response of the germinal vesicle.

Sea water percentage	Diameter of germinal vesicle, microns	Volume of germinal vesicle, cubic microns	Increment per cent	Surface area microns^2	Increment per cent
100	31.0	15,630		3,020	
90	32.4	17,830	14.0	3,300	9.3
80	33.7	20,100	12.7	3,560	7.9
70	35.7	23,190	15.0	4,000	12.3
60	37.3	27,180	17.2	4,370	9.3
50	39.3	31,900	17.3	4,850	11.0
40	41.1	36,500	14.4	5,300	9.3
Total increment		20,870	133.0	2,280	76.0

TABLE III

Osmotic response of the cytoplasm.

Sea water percentage	Cytoplasmic volume	Increment per cent
100	70,370	
90	76,370	8.5
80	82,200	7.6
70	90,310	9.9
60	98,820	9.4
50	108,100	9.4
40	124,000	14.8
Total increment	53,630	76.4

it is of interest to consider the water relations of the cytoplasm separately. From Table III it will be seen that, after the initial thrust of the egg against the dual resisting forces of the membranes, the percentage increase in volume of cytoplasm becomes fairly constant. In the range from 80 to 50 per cent sea water there is a steady increase at the average rate of 9.6 per cent for each 10 per cent of sea water dilution. The 0.4 per cent difference between dilution of the medium and the increase in volume of the cytoplasm may be taken to represent the effect of the mechanical resisting force of the egg surfaces. In view of the constancy of cytoplasmic volume increase, the acceleration shown by the egg as a whole is attributable to the response of the nucleus. The abrupt rise in percentage increase of cytoplasmic volume in going from 50 to 40 per cent sea water is interpretable as an indication of impending injury as the limit of dilution tolerance is approached.

Dilution Tolerance of Spisula sperm and of the Process of Fertilization

For obvious reasons the effect of water upon sperm cannot be studied in the same way as that on eggs. Swimming behavior and also the ability of sperm to activate eggs are taken as indications of the effect. But—even though eggs survive given dilutions and can be fertilized upon return to sea water—it cannot be said that a lack of activation in diluted sea water is not at least partly due to the condition of the egg itself.

Observations showed clearly that the ability of sperm to swim is noticeably inhibited in 70 and in 60 per cent sea water. In 50 and in 40 per cent swimming is limited to occasional short darting movements, but these persist even after one hour of exposure. At least when exposure is brief, about ten minutes, active swimming is resumed by sperm from all dilutions.

When inseminated in diluted sea water, activation of eggs begins to drop off in 70 per cent and is sharply diminished on further dilution. In four experiments sperm were first placed in diluted sea water and ten minutes later eggs were added. Within an hour the first cleavage occurred in the controls, and in the 90 and 80 per cent dilutions, the cleavage in the last being slightly retarded. At this time there was not yet any cleavage of eggs in 70 per cent sea water or below, but various degrees of response were nevertheless shown. 93 per cent of the germinal vesicles had broken down in 70 per cent sea water, 45 in 60 per cent, 4 in 50 per cent, and one per cent in 40 per cent sea water. Four hours later 87 per cent cleavage was found in 70 per cent sea water and 33 per cent in the 60 per cent solution. None had occurred in either 50 or 40 per cent. Six to eight hours after insemination larvae were

swimming actively in the controls and in 90 and 80 per cent sea water; a little sluggishly in the last. In 70 per cent sea water fewer larvae were motile, and these moved only slowly on the bottom of the dish.

The inhibiting effect of water was noticeable also in that cleavage was less advanced with greater dilution. For example, in one experiment at 21° C the two-cell stage was attained in sea water and in 90 per cent sea water within an hour. At this time cleavage in 80 per cent sea water was just beginning; in 70 per cent egg and sperm nuclei had appeared; in 60 per cent no pronuclei were yet in evidence, but 90 per cent of the germinal vesicles had matured; in 50 per cent only 14 per cent of the vesicles had responded, and in 40 per cent only two per cent of the eggs lacked the unmatured vesicle.

In one experiment sperm were exposed to dilution for two hours before eggs were added. One hour later sperm and egg pronuclei had appeared in 90 and in 80 per cent sea water as well as in the controls; none in 70 per cent or below. Examination two and one-half hours later showed, however, that the sperm had not been entirely inactivated, for 17 per cent of the eggs had then cleaved in 70 per cent sea water and one per cent in 60 per cent sea water. Practically all of the uncleaved eggs were in the germinal vesicle stage, i.e. unactivated.

In five experiments sperm were exposed to dilution for ten minutes, eggs then added, and the material restored to natural sea water within an hour. Over 90 per cent of the zygotes from dilutions down to and including 70 per cent sea water cleaved; 65 per cent of those from 60 per cent sea water; 33 from 50 per cent sea water; and 10 per cent from 40 per cent sea water. This demonstrated that sperm could survive all of the dilutions for a brief interval, and further indicated that dilution retarded or inhibited cleavage.

Effect of Water upon Cleavage

The experiments of the previous section differ from those which follow in that, above, insemination took place in diluted sea water and the sperm were exposed to its effect before contact with eggs. In the series now described, in order to study the effect of water after germinal vesicle response had taken place, zygotes were transferred to dilutions ten to fifteen minutes after insemination. The results were uniform in all of eight of such experiments, and one is reported as an example.

At a sea water temperature of 22.8° C 100 per cent germinal vesicle response occurred within ten minutes. Three minutes later samples were transferred into the dilutions. Twenty minutes after insemination polar bodies were

visible on eggs in all solutions down to and including 70 per cent sea water. Fourty minutes after insemination the cytoplasm of eggs in 70 per cent sea water was deeply cut in by a furrow on one side, which subsequently disappeared. In 50 to 55 minutes after insemination cleavage occurred in sea water and in 90 per cent sea water, followed by cleavage in 80 per cent sea water at one hour. At this time sperm and egg pronuclei appeared in the 70 and 60 per cent solutions, but not in 50 or 40 per cent. Observation at two hours after insemination showed 85 per cent cleavage in 70 per cent sea water, in the four-cell stage; and 8 per cent in 60 per cent sea water, in the two-cell stage. There was no cleavage in 50 or 40 per cent sea water.

At one hour after insemination portions of the eggs from each dilution had been returned to natural sea water. One hour after return 90 per cent or more of the eggs from solutions down to and including 70 per cent had cleaved. In eggs from 60 per cent sea water 71 per cent had cleaved, in the four-cell stage. 37 per cent of those from 50 per cent sea water and 17 per cent from 40 per cent sea water had cleaved, in the two-cell stage. Swimming larvae later observed were increasingly sluggish in movement, and less frequent, beginning with zygotes from the 60 per cent dilution. Of material retained under dilution, larvae were active only in the controls and in 90 and 80 per cent sea water. A few also moved sluggishly in 70 per cent, but none below.

Interesting supplementary observations from additional experiments include a case where cleavage was delayed for ten minutes, following a thirteen minute exposure of zygotes to 40 per cent sea water ten minutes after insemination. It was observed also that the cleavage furrow began to cut into these eggs before the pronuclei had been formed. In another experiment, at a temperature of 26° C, portions of a batch of eggs were transferred into dilutions five minutes after insemination. Polar bodies, observed twenty minutes later in the 70 per cent sea water solution, were at least five times the volume of those formed in natural sea water. Forty minutes after insemination pronuclei were evident within zygotes in sea water and in the 90 and 80 per cent solutions. As yet none existed in the 70 per cent dilution, but here a furrow extended from the region of the polar body up to half-way across the egg. At this time the eggs in 60 per cent sea water showed giant polar bodies. In 50 and 40 per cent sea water there was no evidence at all of maturation or cleavage. The controls cleaved 52 minutes after insemination, followed at 55 minutes in 90 per cent sea water and at 57 minutes in 80 per cent sea water. At 63 minutes pronuclei became visible in the 70 and 60 per cent solutions, and at 74 minutes cleavage began in the former. 12

per cent of the zygotes in 60 per cent sea water showed a cytoplasmic bulge with more or less constriction at 90 minutes. Below 60 per cent, aside from osmotic enlargement, the eggs were unresponsive.

The Effect of Water upon Maturation

In four experiments eggs were exposed to 40 per cent sea water for a five minute period at fixed times after insemination, and then returned to sea water. By this means they were flushed with water during different phases of the maturation process. The first exposure was made in from two to fifteen minutes after sperm were added, and subsequent ones at 25, 40 and 50 minutes after insemination. One experiment is cited as an example. In the controls polar bodies appeared 25 minutes after insemination, pronuclei at 40 minutes, and cleavage began at 50 minutes. 98 per cent cleaved.

Experimentally treated eggs were examined 70 minutes after insemination. Of a portion treated with 40 per cent sea water nine minutes after insemination 86 per cent cleaved, and none of the remainder showed injury. Of those flushed with water 25 minutes after insemination—when the polar bodies had already appeared—27 per cent cleaved, 21 per cent were uncleaved, and 52 per cent had cytolyzed. The cytolyzed egg existed as a shrunken, opaque mass, within an extended vitelline membrane. 85 per cent cleaved of eggs exposed 40 minutes after insemination, and none cytolyzed. At fifty minutes the eggs responded similarly, with 92 per cent cleavage. In two experiments there was evidence of cytolysis at 40 and 50 minutes after insemination but this was at the low level of 4½ per cent.

DISCUSSION

In these experiments water was used as an experimental tool to determine the physical and physiological limits of tolerance of gametes when they are mitotically quiescent before activation, and when they are mitotically active after activation, during the maturation process; also of the organism during its period of greatest mitotic activity, in the earliest cleavage stage.

The unfertilized *Spisula* egg enlarges progressively in sea water of increasing dilution, thus manifesting its osmotic properties. If the egg were a perfect osmotic system then the response to dilution should follow the Boyle-van't Hoff Law that $PV = K$. The product of the cell volume (V) and the relative osmotic pressure of the sea-water (P) should remain at a constant figure (K). Experimentally such is not the case, either with the *Spisula* egg, or other

eggs. Additional factors, other than pressure and volume, must therefore be involved.

Lucke and Ricca [10] suggested that a fraction of osmotically inert material is present in the cytoplasm. When allowance was made for this postulated material b then a closer adherence to the van't Hoff Law was attained; when modified into the form $P(V-b)=K$. For the oyster egg b equalled 44 per cent of the cell volume in sea water. On the other hand, Leitch [8] had found little value in assuming a non-solvent fraction in the *Echinometra* egg.

When the volume of the egg is known in different osmotic pressures of the medium, then b can be found from the relationship $P(V-b)=P_1(V_1-b)$. For example, in the present experiments the volume of the *Spisula* egg is 86,000 cubic microns in undiluted sea water and 160,500 in 40 per cent sea water. Substituting these values in the above equation we obtain $1(86{,}000-b)=0.4(160{,}000-b)$, and b has a value of 33,400 cubic microns, or 38.9 per cent of the volume of the egg in sea water. This value is reasonably close to that for the oyster egg. However, when similarly calculated for other dilutions, b for the *Spisula* egg becomes 37.3 per cent in 50 per cent sea water, 30.5 per cent in 60 per cent sea water, 27.2 per cent in 70 per cent sea water, 23.2 per cent in 80 per cent sea water, and 12.8 per cent in 90 per cent sea water. If these values are plotted and extrapolated b acquires a value of about 12 per cent in natural sea water, and would vanish if the egg were placed in concentrated sea water of 126 per cent.

It appears unlikely that osmotically inactive materials should increase in amount with dilution, but such a viewpoint has been advanced by Kamada and Yamamoto [7]. That there is some unusual condition operating in the *Ceratocephale* egg, with which these investigators worked, is indicated by the fact that the nucleus does not begin to respond osmotically until about ten minutes after the egg has been exposed, as found by Kamada [6].

In visualizing the egg as an osmotic system it seems obvious that, as the cell enlarges, the physical resistance of stretched surface membranes should not be disregarded as a factor which affects volume at equilibrium. It is logical to assume that the forces which resist swelling become increasingly effective as the tension increases, up to the point of irreversible injury and rupture of the membranes. In any given dilution of sea water a volume equilibrium should therefore be attained before the size to be expected from the van't Hoff Law. This would account for the increasing value of b with dilution. Conversely, in concentrated sea water b should disappear at the point of plasmolysis. It is interesting that Churney [3] has observed that *Arbacia* eggs lose their spherical shape in 131 per cent sea water. This con-

centration, where the egg membranes of *Arbacia* are no longer under stretch tension, is very close to the concentration of 126 per cent, determined above for *Spisula*, at which *b* vanishes. The natural conclusion that *b* is related chiefly to the physical properties of the egg surface appears to be supported by the work of Bialaszewicz [2], who found that egg cells obey the van't Hoff Law when shrinking in hypertonic solutions, but do not when swelling.

Evidently the situation is not the same for the nucleus as for the egg as a whole, for the germinal vesicle swells more than is to be expected from the van't Hoff Law. While, as shown in the data, the cytoplasm responds at a fairly constant rate to dilution of the medium, the germinal vesicle does so with increasing acceleration. This may be an important consideration with regard to the effect of water upon mitotically active eggs.

When eggs are inseminated in diluted sea water, maturation phenomena and cleavage are delayed in proportion to the dilution and, finally, in the lowest dilutions, i.e. in 50 and 40 per cent sea water, they are completely or almost completely inhibited. This effect is reversible to a large extent for, when eggs inseminated in diluted sea water are returned within an hour to natural sea water cleavage rates are much higher. For example, in 70 per cent sea water the first cleavage was delayed three or four hours and eventually occurred in 87 per cent of the eggs. If returned to natural sea water within an hour after insemination there was no apparent lowering of the cleavage percentage below that of the controls. In eggs returned from the 60 per cent dilution about twice as many cleaved as compared with those allowed to remain. One-third of the eggs from 50 per cent sea water cleaved upon return to natural sea water, as contrasted with no cleavage when allowed to remain. Even when returned from 40 per cent sea water, 10 per cent of the eggs cleaved. The evidence shows also that cleavage of zygotes formed in natural sea water is progressively delayed and inhibited when the zygotes are transferred to diluted sea water after insemination.

Particularly interesting is the effect of dilution upon the polar body stage of the maturing egg. While the unfertilized egg is 87 per cent larger in 40 per cent sea water and the nucleus more than twice its usual size, the polar body, even in 70 per cent sea water, swells to several times its normal volume. The cytolysis of fertilized eggs in 40 per cent sea water, when exposed in the polar body stage, seems correlated with and caused by the extraordinary swelling of the latter.

The destructive effect of water at the time of polar body formation and its inhibitory effect upon maturation and cleavage may be interpreted as different phenomena.

In the case of the polar body, it seems that the relatively large amount of nuclear material in proportion to cytoplasm brings about an unusual amount of osmotic swelling. This is in harmony with the finding that the osmotic response of the nucleus of the unfertilized egg is much greater than that of the cytoplasm. As a consequence, the polar body—probably prior to the time when its cytoplasm has become sealed off from the cytoplasm of the egg—is ruptured, and brings about cytolysis of the egg. Many years ago Lillie [9] discovered that *Arbacia* eggs are highly susceptible to diluted sea water when the cleavage furrow begins to form, but regain their original resistance after completion of the furrow. It is suggested that here too the proportion of nuclear to cytoplasmic material is significant, each cell after cleavage having half the amount of nuclear material which it had in the pre-cleavage phase.

The inhibitory effect of water—involving a delay in the process of maturation and a delay in cleavage—requires a different explanation; one in the nature of a reversible injury, as of narcosis. Swann [13] in an interesting discussion of the mechanism of cell division, points out that narcotics are known as reversible inhibitors of cell division, and that one of the effects of ether in low concentrations is to block the building up of the mitotic figure through a process of solation. That cell division is preceded by mitotic gelation has long been emphasized by Heilbrunn [5], who believes that narcotics dull the "trigger-like action" of the calcium ion in bringing on the gelation. Water, then, in its inhibitory effect upon cleavage, appears to fall into the category of a narcotic, producing an injury by preventing the normal gelation required for mitosis and cleavage. Through the subsequent withdrawal of water it has been shown in this paper that the effect is reversible. Since the delay in cleavage begins in 80 per cent sea water, and is pronounced in 70 per cent we might conclude—using the behavior of the germinal vesicle as a criterion—that the narcotic effect upon mitosis begins when the nuclear volume has been increased about 50 per cent due to the inflow of water. The germinal vesicle itself, in the unfertilized egg, can survive, without permanent injury, an inflow of water which enlarges its volume by 133 per cent. This occurs in 40 per cent sea water, which the zygote can also survive for a period of time, as shown in these experiments.

A final observation, of special interest, is the appearance of a transient cleavage furrow, in eggs in diluted sea water, before the nucleus is ready for division, thus throwing the cortical and nuclear phenomena of cleavage out of coordination under the influence of excess water. This, too, shows the higher responsiveness of the nucleus to water narcosis.

SUMMARY

In a study involving the gametes and the zygote of the surf-clam, *Spisula solidissima*, it has been shown that:

1. Sperm, unfertilized eggs, and activated eggs—except in the polar body stage, can withstand dilution of sea water for as much as 40 parts sea water to 60 parts distilled water.

2. In its osmotic response the egg has been found to be able to survive a volume increase of 87 per cent and an increase in surface area of 51.5 per cent.

3. The germinal vesicle of the unfertilized egg is osmotically greatly more responsive than the cytoplasm.

4. The germinal vesicle can survive a water intake which increases its volume by 133 per cent, and its surface by 76 per cent.

5. Excess water within the fertilized egg produces a reversible injury, in in the nature of a narcosis, which delays or inhibits maturation and cleavage.

6. The activated egg in the polar-body phase is highly sensitive to and is destroyed by an amount of excess water which is tolerated before and after this phase.

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SOME RELATIONS BETWEEN GROWTH AND CARBOHYDRATE METABOLISM IN TISSUE CULTURES[1, 2]

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Study of the metabolism of tissues grown *in vitro* provides an excellent opportunity for the investigation of relations between growth and metabolism in proliferating cells. The present studies concern the relative importance of glycolytic and oxidative glucose breakdown for the multiplication of chick embryonic tissues *in vitro*.

Early reports in this field present conflicting data for growth in the absence of oxygen, ranging from no growth to normal growth. Burrows [5, 6], Wright [20], and Lipmann [14] using chick embryonic tissues, and Mottram [15] with rat tissues observed no growth under anaerobiosis, whereas Wind [19] and Gomirato [10] with chick embryonic tissues demonstrated some growth; Laser [13] employing chick embryonic tissues found normal growth. On the one hand negative results were probably in some cases due to the use of stationary cultures and solid media, in which toxic levels of lactic acid accumulated around the cells. On the other side areal measurements as an indication of growth are unreliable. Also in some instances white phosphorus, used to obtain an anaerobic system, may have been toxic to growth. In none of these early studies were specific determinations of the metabolites made. It is, therefore, not surprising that so many different conclusions concerning the relative importance of glycolysis and respiration for growth have been reached.

The present investigation determines the effect of nitrogen, oxygen, and

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[2] Preliminary reports have been given at the annual meeting of Tissue Culture Association, Galveston (Texas), April 1954, and at the Third International Congress of Biochemistry, Brussels, August 1955.

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cyanide on (*a*) the consumption of glucose and the production of lactic and pyruvic acids, and (*b*) the growth of chick embryonic cells measured in terms of protein synthesis.

EXPERIMENTAL METHODS

The quantitative roller tube method of Gerarde, Jones and Winnick [9] has been employed, using 15-day chick embryo lung and intestine. The medium used was 25 per cent chick embryo extract (EE) in balanced salt solution, with the initial pH adjusted to 7.8–7.9. The salt solution (Table I) is a modified Tyrode solution containing increased amounts of bicarbonate and phosphate so as to obtain maximum buffer capacity.

Incubation was at 37°C, the medium was changed routinely at 1–2 day intervals, and cultures were maintained for as long as 2 weeks. The modified salt solution had the advantage of prolonging the maximum incubation time under anaerobic conditions from about 15 hours to over 24 hours. The pH of the medium removed at each change was recorded as a means of following the activity of the cultures. Samples of culture medium for chemical analysis were taken following initial and final intervals of incubation during the experiments. In order to minimize the effects of injury caused in the preparation of the tissues, all cultures were incubated under control conditions (25 per cent EE and air atmosphere) for 2 days following the explantation and preceding the experiments.

Synthesis of protein was determined by isolation and weighing of protein in initial cultures and in tissues after incubation [9]. In later experiments this method was replaced by a colorimetric method [16, 4]. For brevity the term "growth" is used here instead of "synthesis of protein", regardless of whether the protein is of intracellular or extracellular nature, since total protein is determined.

The ultramicrochemical methods used for the determination of glucose, lactic acid, and pyruvic acid in this study have been published elsewhere [1, 2, 3].

TABLE I

Composition of Tyrode solution and modified solution of greater buffer capacity.

	Tyrode solution		Modified solution	
	mg/100 ml	m*M*/1	mg/100 ml	m*M*/1
NaCl	800	137.1	765	131.1
KCl	20.0	2.7	37.2	5.0
$CaCl_2 \cdot 2H_2O$	26.5	1.8	16.2	1.1
$MgCl_2 \cdot 6H_2O$	10.0	0.5	—	—
$MgSO_4 \cdot 7H_2O$	—	—	12.3	0.5
$NaH_2PO_4 \cdot H_2O$	5.0	0.4	166	6.0
$NaHCO_3$	100	11.9	202	24.0
Glucose	100	5.6	100	5.6

RESULTS

In Table II results with 15-day lung cultivated aerobically in 25 per cent embryo extract prepared with each of the two salt solutions are compared. Since there were no significant differences in carbohydrate metabolism, protein synthesis, and cellular appearance, the modified salt solution of greater buffer capacity was adopted for use in further experiments.

In a study of the effect of the concentration of glucose in the medium the initial levels of glucose were varied from 1 to 5 mg/ml. Marked effects on growth and metabolism of the cultures were observed. Table III presents an experiment with lung which shows that increasing the concentration of glucose caused a marked and regular increase in glucose consumption and lactic acid production while growth decreased. At the same time the percentage of glycolysis (ratio of lactic acid production to glucose consumption, both in mg/day) increased. No effect on the production of pyruvic acid was noted. Microscopic observations of the cells showed an increasing cellular granularity with increasing concentrations of glucose. Cultures of intestine gave similar results. Unless otherwise indicated, a level of 1 mg/ml glucose was used in subsequent experiments.

In order to study varying atmospheres of gas, culture tubes were flushed for one minute with a rapid stream of nitrogen or oxygen. The medium was changed daily, and determinations were made at the end of the 1st and 8th days.

TABLE II

Metabolism and growth of chick embryonic lung cultivated in Tyrode solution and modified solution.[1]

	Glucose uptake mg/day	Lactic acid production mg/day	Pyruvic acid production mg/day	Glycolysis[2] %	Growth[3] %	pH[4]
Tyrode solution . . .	1.96	−0.06	0.030	0	478	6.94±.04
Modified solution . . .	2.07	−0.07	0.041	0	480	7.09±.06

[1] Glucose concentration in the medium was 1.25 mg/ml; samples of the medium for analysis were removed after the 7–9th day period of incubation.

[2] Glycolysis is represented as the ratio of lactic acid production to glucose consumption, both in mg/day.

[3] Growth is represented as percentage increase in protein over initial values.

[4] pH figures represent average values over the total period of cultivation.

TABLE III

Effect of glucose concentration on metabolism and growth of lung.[1]

Glucose conc. mg/ml	Glucose uptake mg/day		Lactic acid production mg/day		Pyruvic acid production mg/day	
	0–2 days	6–8 days	0–2 days	6–8 days	0–2 days	6–8 days
1.0	1.90	2.03	0.18	− 0.10	0.032	0.023
2.0	3.10	2.59	1.11	0.63	0.052	0.078
3.0	3.26	3.06	1.41	1.28	0.022	0.050
5.0	4.30	3.98	1.54	1.38	0.026	0.061

Glucose conc. mg/ml	Glycolysis %		Growth %	pH	Utilization %	
	0–2 days	6–8 days			0–2 days	6–8 days
1.0	10	0	136	7.19 ± .05	80	86
2.0	36	24	63	7.05 ± .12	71	59
3.0	43	42	54	6.91 ± .04	55	52
5.0	36	35	2	6.82 ± .03	41	38

[1] The explanatory footnotes 2, 3 and 4 in Table II apply here.

In Fig. 1 an 8-day experiment with lung cultivated under nitrogen is represented; the carbohydrate metabolism was fully anaerobic, while the consumption of glucose increased somewhat, although less during the final than the initial period. As seen by the pH curves, the activity remained constant throughout. Growth was only slightly less than in the controls. Intestine (Fig. 2) gave comparable results. In both types of tissue the cells under nitrogen became more granular toward the end of the incubation period, and migration was markedly less in nitrogen than in the controls. The epithelial cells in anaerobic lung cultures were smaller than those in aerobic cultures, while the nuclei also were smaller, and often located near the very pronounced cell walls. The epithelial cells and nuclei in anaerobic intestine cultures were only slightly shrunken.

Under oxygen (Fig. 3) the carbohydrate metabolism of lung was shifted partly to the anaerobic side, and after 8 days no metabolic activity took place. Judging from the pH curves, it would appear that the cells became inactive after 4–6 days, with a loss instead of a gain in protein. Intestine

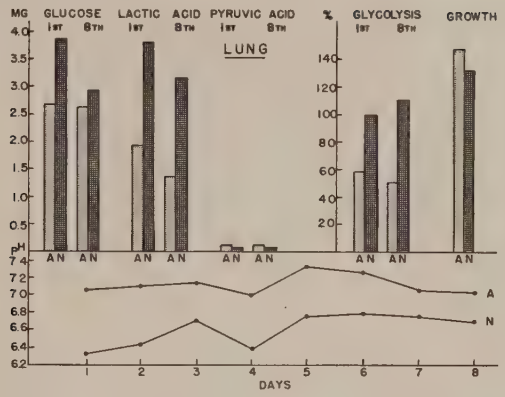

Fig. 1. Growth and metabolism of lung in an atmosphere of nitrogen. The bars represent air over A, and nitrogen over N.

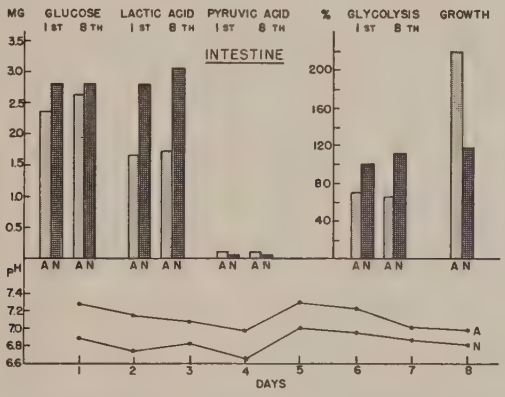

Fig. 2. Growth and metabolism of intestine in an atmosphere of nitrogen. The bars represent air over A, and nitrogen over N.

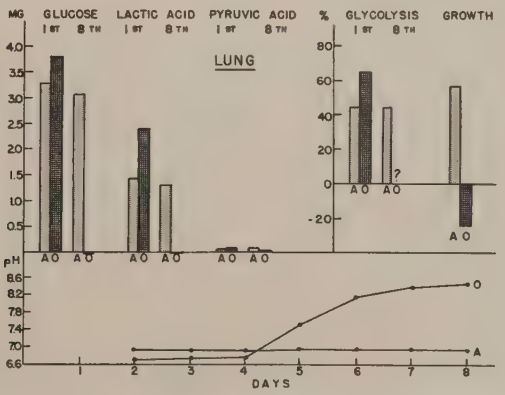

Fig. 3. Growth and metabolism of lung in an atmosphere of oxygen. The bars represent air over A, and oxygen over O.

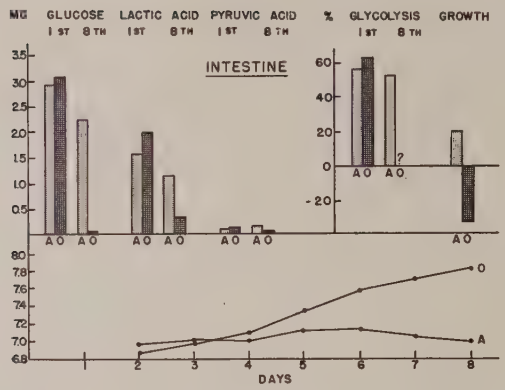

Fig. 4. Growth and metabolism of intestine in an atmosphere of oxygen. The bars represent air over A, and oxygen over O.

HCN mM/L	GLUCOSE MG 0-2 DAYS	GLUCOSE MG 8-10 DAYS	LACTIC ACID MG 0-2 DAYS	LACTIC ACID MG 8-10 DAYS	PYRUVIC ACID MG 0-2 DAYS	PYRUVIC ACID MG 8-10 DAYS	GLYCOLYSIS % 0-2 DAYS	GLYCOLYSIS % 8-10 DAYS	GROWTH %	GROWTH % OF CONTROL
0	2.11	2.64	0.51	0.77	0.032	0.051	24	29	234	100
1/8	2.88	2.56	1.38	0.67	0.001	0.028	48	26	160	68
1/4	2.93	2.18	2.54	1.73	0.006	0.007	87	80	122	52
1/2	2.97	0.14	2.81	0.23	0.004	0.020	95	—	64	27

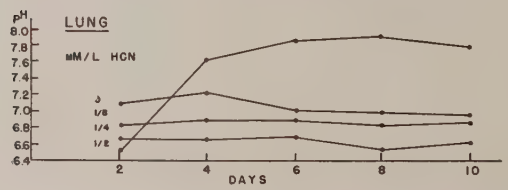

Fig. 5. The effect of cyanide on growth and metabolism of lung.

HCN mM/L	GLUCOSE MG 0-2 DAYS	GLUCOSE MG 8-10 DAYS	LACTIC ACID MG 0-2 DAYS	LACTIC ACID MG 8-10 DAYS	PYRUVIC ACID MG 0-2 DAYS	PYRUVIC ACID MG 8-10 DAYS	GLYCOLYSIS % 0-2 DAYS	GLYCOLYSIS % 8-10 DAYS	GROWTH %	GROWTH % OF CONTROL
0	1.90	1.03	0.33	0.10	0.042	0.036	17	10	158	100
1/8	2.13	1.62	1.23	0.90	0.028	0.042	58	56	111	70
1/4	1.75	0.70	1.10	0.23	0.020	0.027	63	33	29	18
1/2	2.11	0.26	1.88	0.15	0.009	0.019	89	—	28	18

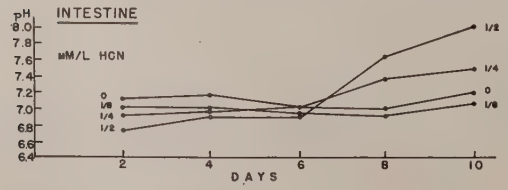

Fig. 6. The effect of cyanide on growth and metabolism of intestine.

(Fig. 4) was inactivated somewhat more gradually. Paralleling the rise in pH the cells of both tissues became very granular.

To examine the effect of cyanide in this system, potassium cyanide was added to the medium in final concentrations of 1/8, 1/4, and 1/2 m*M*/1. Fig. 5 presents the results for lung. Increasing the concentration of cyanide shifted the carbohydrate metabolism more and more to the anaerobic side, with almost complete glycolysis in $^1/_2$ m*M*/1 cyanide in the early period before death of the cells at 4–6 days. Growth decreased with increasing amounts of cyanide, but was not inhibited completely until concentrations were $^1/_2$ m*M*/1 or above. It is important to note here the decreased pyruvic acid production. In higher concentrations of cyanide, 1 and 2 m*M*/1, cultures of lung died in 3 days, with an average growth of 28 per cent and practically 100 per cent glycolysis during the first 3 days. Intestine (Fig. 6) was more gradually inactivated by increasing concentrations of cyanide; however, results similar to lung were observed. Migration of cells in both lung and intestine was inhibited greatly in the higher cyanide concentrations.

DISCUSSION

Under the conditions of these experiments embryonic tissues cultivated *in vitro* in an air atmosphere had a predominantly respiratory metabolism; only when the cells could afford to be less efficient—in the case of high glucose levels—did the percentage of glycolysis increase. This is in contrast to previous investigations by Krontowski [12] and Demuth [7], which would indicate that the glycolytic process was more important than the respiratory glucose utilization as an energy-yielding process for chick embryonic tissues.

When these tissues are cultivated under 100 per cent nitrogen, the metabolism becomes completely glycolytic. The average percentage glycolysis for lung was 93 ± 7 per cent[1] (5 experiments), and for intestine 97 ± 15 per cent (3 experiments). Under these conditions both tissues show growth: an average of 82 ± 15 per cent of growth under aerobic conditions for lung and 73 ± 26 per cent for intestine. In addition there is a somewhat increased glucose uptake. Thus, these tissues can maintain their synthetic ability almost completely with the free energy derived from glycolysis alone. This is in agreement with the finding by Zamecnik and Keller [21] that amino acid incorporation

[1] Standard deviation of the observations, defined by the formula: S.D. $\sqrt{\frac{\Sigma (x_i - \bar{x})^2}{n-1}}$, where x_i represents an observation, $\bar{x}$ the average of the observations, and n the number of observations.

in the proteins of a cell-free rat liver homogenate can take place under anaerobic conditions as long as ATP is generated. Since the free energy of the glycolytic process is only about $^1/_{15}$ that for the respiratory process per molecule of glucose utilized, it is clear that the total free energy available for the nitrogen cultures was much less than for the air cultures. This lack of free energy could be responsible for the decreased migration of cells from explants cultivated in nitrogen.

Cyanide, present in the medium in concentrations sufficient to inhibit respiration completely, also partly inhibited glycolysis; the latter effect becomes considerable at slightly higher concentrations and after 3–4 days only. Laser [13] also reported this effect. This should be a warning against the indiscriminate use of cyanide as a respiratory inhibitor. The decreased production of pyruvic acid in the presence of cyanide—also noticeable in the nitrogen cultures (Figs. 1 and 2)—can probably be explained in terms of reduction of pyruvic acid to lactic acid and other compounds [11].

Oxygen inhibits the respiratory enzyme systems first and then gradually starts inhibiting the glycolytic system, and thereby growth. Demuth [7, 8] has reported an inhibition of growth by pure oxygen. This effect is probably due to the oxidation of essential SH-groups in the pertinent enzymes. The work of Stadie and Haugaard [17, 18] suggests that the initial effect of the oxygen is an inactivation of succinic dehydrogenase due to this effect, while later on apparently even glycolytic enzymes with essential SH-groups are affected.

SUMMARY

1. The effects of nitrogen, oxygen and cyanide on the carbohydrate metabolism and the growth in terms of protein synthesis have been investigated in cultures of 15-day chick embryonic lung and intestine.

2. On cultivation under air respiration predominates over glycolysis, but increase of the glucose level in the culture medium raises the percentage glycolysis considerably.

3. On cultivation under 100 per cent N_2 carbohydrate metabolism becomes completely glycolytic, while growth is almost normal. The tissues remain alive and active for the duration of the experiment (8 days).

4. In cyanide (0.25–0.50 m*M*/l) with respiration largely inhibited, protein synthesis is severely decreased. The tissues become inactive at 4–6 days.

5. On cultivation under 100 per cent O_2 respiration is markedly decreased, while protein is lost from the explants. The tissues become inactive at 4 days.

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CALCIUM IN AGING AND FERTILIZED FROG EGGS

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Lansing [5, 6], Simms and Stolman [14] and others have shown that calcium increases with age in a variety of tissues of many organisms. The fact that Lansing [4] could prolong life of rotifers by decreasing the concentration of calcium in the medium and by exposing the organisms to citrate, and that Schechter [10, 11] inhibited aging in invertebrate eggs by decreasing the calcium in the medium suggest that accumulation of calcium in tissues may be a causative factor in the aging process.

McCay [8] and others have shown that prolongation of growth greatly extends the life span, and the concept has arisen that aging occurs most rapidly in cells that are not undergoing mitosis. That calcium accumulates to a greater extent in slower growing than in faster growing tissue was shown by Lansing, Rosenthal and Au [7] who found less calcium in hyperplastic and neoplastic epidermis than in normal epidermis. The accumulation of calcium in slowly growing and in aging tissues, and the inhibition of aging by growth and a decrease in the amount of calcium available to cells support the hypothesis that accumulation of calcium in cells which are not dividing may be a factor resulting in aging.

The present investigation was carried out to determine if calcium accumulates in single aging cells which are not dividing, and which are not subject to influences of other organs or tissues. Unfertilized frog eggs were chosen for study because they are essentially cells of the adult frog which age under natural conditions, subject to minimal trauma. They differ from somatic cells in being haploid, totipotent and containing yolk but it has not been shown that these factors are related to aging. Presence of yolk is an advantage for it is then known that changes noted with the age of cells are not due to lack of provision of essential nutrients.

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METHODS

Female *Rana pipiens* which had been maintained at 4° C were injected with frog pituitaries as described by Rugh [9]. After 45–48 hours at room temperature, eggs were expressed into standard Holtfreter's solution containing 0.005 per cent sulfadiazine to inhibit bacterial growth. Half the eggs were placed in a sperm suspension in the same medium to test the viability of the eggs, and to have actively growing cells, the calcium content of which could be compared to that of the aging unfertilized eggs. Although it was shown by Gregg and Ballantine [1] that the nitrogen content of frog embryos does not vary significantly from time of ovulation through the period covered in this investigation, developing embryos cannot be considered controls for unfertilized eggs due to the constantly increasing ratio of cell surface and nuclear volume to cytoplasmic volume, and factors of differentiation in the embryos.

For each experiment, eggs were obtained from a single frog. Cultures were maintained at room temperature and the media were changed twice daily. At various time intervals eggs and embryos were removed, the jelly coats were removed with scalpel and forceps and the organisms were stored at −10° C in a mixture by volume of two parts acetone and one part methanol. For calcium determinations each egg was homogenized in 0.3 ml of the acetone–methanol mixture. The mixture was centrifuged and the supernate removed. The residue was washed with another 0.3 ml portion of the acetone–methanol mixture and the supernates were combined. The residue was similarly extracted with two 0.3 ml portions of distilled water. The fat-soluble, water-soluble and insoluble fractions were then ashed by the method of Kapuscinski

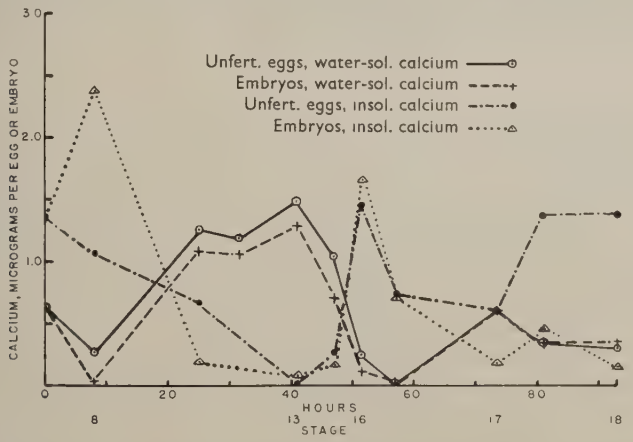


Fig. 1. Calcium in water-soluble and insoluble fractions of unfertilized eggs and embryos. Each point represents average of three separate determinations. Stages refer to stage of development of embroyos as described by Shumway [13].

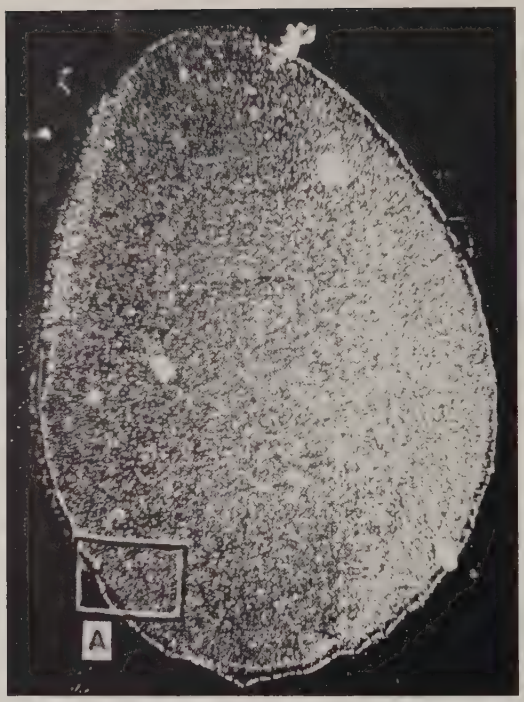
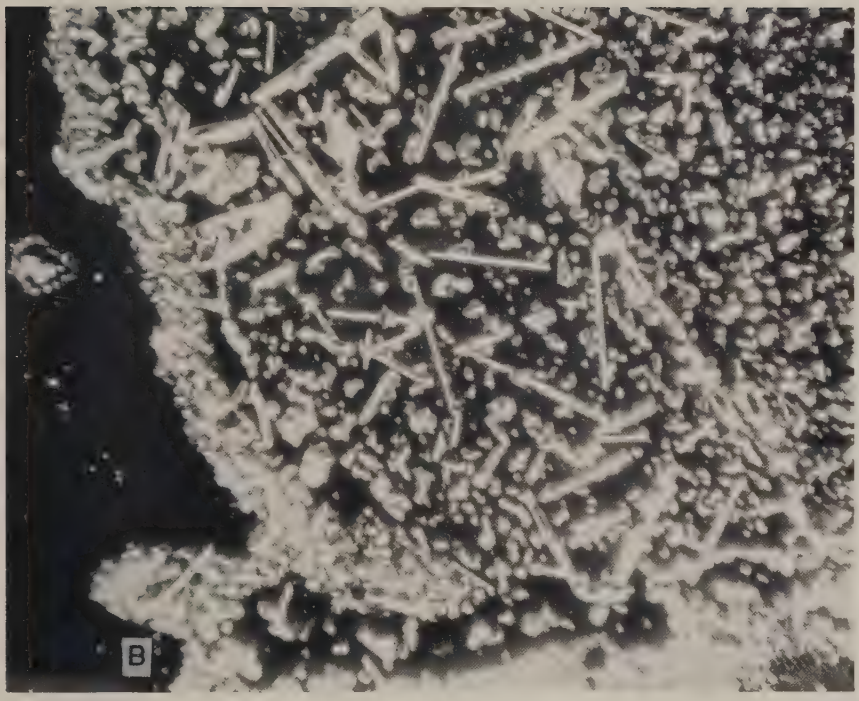


Fig. 2. Histological sections cut at 5μ and ashed. *A* (left), unfertilized egg at 8 hours. *B* (p. 643), outlined area of *A* under higher power after treatment with HCl and H_2SO_4, showing needles of gypsum. Dark-field illumination.

et al. [2]. The calcium was determined by the micromethod of Kuttner and Cohen [3], modified in that determinations were made with a spectrophotometer at 670 mμ instead of a colorimeter.

In order to determine if changes in calcium occurred within the organism, in the vitelline membrane, or in some of the jelly coat which was not completely removed, a histological study was carried out. When embryos and unfertilized eggs were removed for calcium determination, eggs and embryos with jelly coat intact were removed and treated according to the microincineration method of Scott [12]. The method was varied in that the organisms were fixed in a 10 per cent aqueous solution of formalin and dehydrated through 70 per cent and 95 per cent ethanol in order to wash out calcium ions which had diffused into the jelly coat from the medium. Since practically no calcium was detected in the acetone methanol extractable fraction, it was believed most of the calcium determined chemically would be in a form fixed by the 10 per cent formalin. Sections stained with hematoxylin and eosin were alternated with those ashed. The ash was tested for calcium by adding a minute drop of 0.1*N* HCl followed by a drop of 0.1*N* H_2SO_4 and observing the formation of characteristic needle crystals of gypsum.



RESULTS

Fig. 1 shows the results of determinations done on eggs obtained April 19, 1955. Only occasional traces of calcium were detected in the fat-soluble fraction and these were not considered significant. Except for the insoluble fractions at 8 hours and after 57 hours, there was an unexpected agreement in calcium levels between embryos and unfertilized eggs at different times. The roughly inverse relationship of the curves for calcium in the water-soluble and insoluble fractions suggests much of the variation is due to interchange of calcium between water-soluble and insoluble fractions rather than exchange with the medium. By 93 hours, calcium in the insoluble fraction of unfertilized eggs was about 9 times that in the embryo.

Some preliminary studies yielded results differing slightly from those shown in Fig. 1. In determinations made on eggs obtained in February 1955

and cultured in slightly diluted Holtfreter's solution, the water-soluble fraction contained most calcium from 0 to 28 hours, at which time it decreased as calcium in the insoluble fraction rose. In that experiment the water-soluble calcium did not attain the level it did in the experiment represented by Fig. 1 and its decrease was not enough to account for the increase in the calcium in the insoluble fraction, indicating calcium must have been taken into the insoluble fraction from the medium. Thus, season, calcium concentration in the medium, and such factors as time between pituitary injection and expression of eggs may affect the time and extent of changes in calcium levels.

In the incinerated sections (Fig. 2) very little ash and practically no calcium was detected in the jelly coats or vitelline membranes of any of the sections. An eosinophilic granular material occasionally seen between the egg and vitelline membrane was poor in calcium. Throughout the experiment calcium seemed to be more concentrated at the periphery of both eggs and embryos.

DISCUSSION

Undoubtedly many different reactions involving calcium occur in embryos and unfertilized eggs. This study yields a view of the sum of these reactions and the data do not justify an attempt to associate changes in calcium levels with any particular aspect of metabolism or differentiation. The fact that changes in calcium levels of unfertilized eggs parallel those in the developing embryos suggests that both unfertilized egg and embryo undergo some of the same metabolic changes and that the unfertilized egg is not a resting cell.

Of interest in regard to cellular aging is the increase in insoluble calcium from 57 to 93 hours in unfertilized eggs as compared to the embryos. Though there is very little difference in nitrogen content between unfertilized eggs and Shumway stage 18 embryos [1], the insoluble calcium of the unfertilized eggs 93 hours old is approximately 9 times that of stage 18 embryos of the same age. This observation is consistent with the theory that accumulation of insoluble calcium compounds is associated with, and may be a cause of cellular aging.

It is planned in future studies to test the following hypothesis: when cells stop growing and dividing they are still provided with a substance (or substances) previously required for growth or mitosis; this substance (or substances) is not able to be utilised and forms insoluble compounds with calcium.

SUMMARY

Fat-soluble, water-soluble and insoluble fractions of unfertilized eggs and developing frog embryos were analyzed for calcium at various times up to 93 hours. Sections of the eggs and embryos were tested for calcium by the microincineration method. No significant amounts of calcium were found in fat-soluble fractions. Changes in water-soluble and insoluble fractions suggested an interchange between the fractions, and showed many similarities between unfertilized eggs and developing embryos. By 93 hours the insoluble calcium per unfertilized egg was about 9 times that in the embryo. The possible significance of calcium in cellular aging is discussed.

The author is grateful to Drs. Lowell Orbison and Melvin Schoenberg for their interest in, and support of this study, and to Miss Kate Gruen for preparation of histological sections.

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PAST HISTORY AND PATTERN OF SEROTYPE TRANSFORMATION IN *PARAMECIUM AURELIA*[1]

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Sonneborn has shown that certain ciliary antigen differences in *Paramecium aurelia*, variety 4, persist indefinitely in a single environment, but that conjugation between diverse serotypes fails to result in the subsequent identity of the two exconjugant clones [9]. Since identical zygote nuclei are formed in each member of a conjugating pair, these observations constitute *prima facie* evidence for cytoplasmic genetic determination.

Extensive analyses have strengthened this original inference, but have also revealed differences between serotype inheritance and the cytoplasmic inheritance of the killer and mating-type traits [10]. Unlike the killer trait, no independently-mutable, sub-cytosomal units have been implicated. Unlike the mating-type trait, serotype may be readily altered in the absence of nuclear reorganization. A most striking attribute of these otherwise stable serotypes is, in fact, the ease with which they may be caused to undergo rapid and massive transformation to another serotype by appropriate environmental change (e.g. of temperature or feeding conditions) or by treatment with corresponding antiserum [9, 14].

The possibility that common principles underlie serotype determination and the cellular differentiation of a multicellular organism has been emphasized elsewhere [12] and has stimulated further investigation.

A particularly economical model of serotype determination is one of a system in "flux-equilibrium" as suggested by Delbrück [2]. On such a model, the self-perpetuating entity ("plasmagene") is nothing less than the entire cytosome, or some diffuse fraction thereof. Perpetuation of diverse serotypes in a common environment is due to the inhibition by each antigen, when in high concentration, of processes leading to the formation of other antigens.

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Changes in temperature or feeding conditions induce transformation by selectively favoring certain antigen-forming reactions; antiserum transforms by directly blocking the postulated inhibition.

The present investigation is concerned with the effects of past environments, specifically feeding conditions, upon the frequency and kind of transformation induced by a standard antiserum exposure. Immediate and distant past history are shown to influence transformation pattern in such a way as to necessitate a reexamination of the simple "flux-equilibrium" model.

MATERIALS AND METHODS

Stock 47, variety 4, was studied; this constitutes the progeny of a single isolation from nature. This stock belongs to the same variety as stocks 51 and 29 and exhibits a similar array of serotypes, namely *A*, *B*, *C*, *D*, *E*, and *J*. Three of these (*A*, *C*, and *D*) are stable at a wide array of temperatures and feeding conditions; three (*B*, *E*, and *J*) are very unstable under all conditions studied [7]. Antisera against the homologous *A*, *B*, *C*, *D*, and *E* serotypes of stock 51, against serotype *J* of stock 29, and against serotype *D* of stock 47 were available and were used rather than resorting exclusively to antisera obtained directly against stock 47 serotypes. Except where specifically noted, growth was at 27° C. The culture medium consisted of a fresh population of *Aerobacter aerogenes* growing in a baked lettuce infusion, adjusted to pH 6 before use [11].

Standard feeding conditions (as for Sonneborn, [11]) allowed for only one doubling of the population per day. This was accomplished by discarding daily half of the culture and substituting the same volume of fresh culture medium. See Preer [5] for a discussion of this method of limiting growth rate. Standard cultures are then in a condition of mild starvation just before each daily feeding. Two deviations from the standard feeding conditions are considered here. The first, more severe starvation, was brought about by simply allowing a standard population to stand without feeding. The second, unlimited growth, was brought about by providing a constant excess of food. The mean growth rate of the latter populations was between 4 and 5 fissions per day, so that daily feedings consisted of approximately 25-fold dilution with culture medium. Peak daily population density fluctuated between about $^1/_8$ and $^1/_{32}$ of maximum.

The standard antiserum exposure for the induction of transformation was as follows. Thirty animals were removed from a population, washed through three successive drops of 1/10 Ringer's solution in a depression slide, and collected together in the last. To this volume (ca. 0.05 ml) was added an equal volume of corresponding antiserum and the mixture was incubated at 27° C in a moist chamber. For serotype *D*, the effective antiserum dilution in the treatment chamber was 1/200 and the duration of exposure was 30 minutes, or about twice the immobilization time. Following exposure, the chamber was flooded with culture medium and each animal quickly isolated into a separate depression chamber with enough food for more than two days of unlimited growth. Tests of quantity and quality of transformation were made at some time after the recovery of motility. If these tests were made before the division

of the recovered animal, it was of course necessary to test first with one antiserum, then with another. If, however, the recovered animal had been allowed to produce a clone, all or part of its progeny could be tested simultaneously with several different antisera. In general, controls were exposed to 1/10 Ringer's solution, but otherwise were treated in the same manner as animals exposed to antiserum; only a few of the control samples were treated with non-homologous antiserum. See Sonneborn and LeSuer [14] for justification of this general use of Ringer's treated animals as controls.

The standard procedure for testing serotype was to use a solution of antiserum four times more concentrated than is necessary to immobilize the corresponding serotype in two hours. This immobilization titer on stock 47 animals was 1/800 for all antisera except anti-*A*, which had a titer of 1/1600. Tests were read after two hours at 27°C.

RESULTS

Immediate past history and transformation pattern.—A culture of serotype *D* was maintained under standard feeding conditions for a period of 120 days. During this time, repeated subcultures were made and these were allowed either to starve or to multiply in the presence of excess food. After varying intervals of time, samples of these subcultures and of the parental culture were exposed to antiserum and the transformation pattern examined 48 hours after treatment. Table I compares the patterns exhibited by starved cultures, and by cultures which had been presented with excess food for a few days, with the pattern characteristic of the standard parental culture. Controls, not exposed to antiserum, showed negligible mortality and transformation and are not included.

Three aspects are considered. The first of these, survival, is markedly affected by starvation for more than three days and is probably affected by the moderate starvation here considered. This is more troublesome than surprising. The second aspect, frequency of transformation among survivors, is clearly different for each of the three types of populations and would appear to be a function of the degree of inanition. The third aspect, frequency of transformation to serotype A among transformed clones, is clearly higher in the starved series than in the standard, but a difference between the standard and excess food series is not detectable. Almost all additional transformation was to serotype *B*. Although these data demonstrate the influence of past environmental conditions upon the "competence" of *Paramecia* to react to antiserum, the full significance of the observations depends upon the answers to several additional questions.

The frequency of transformation and the relative frequency of transformation to serotype *A* have been calculated on the basis of survivors. Could the

TABLE I

Effects of prior feeding conditions upon the pattern of transformation of serotype *D* following standard antiserum exposure. Part *A* compares standard populations with populations starved for 2 or 3 days. Part *B* compares standard populations with populations in excess food for 1, 2, or 3 days. Pairing is on the basis of simultaneous antiserum exposure and recent common origin of animals exposed. The chi-squares measure dispersion in the 2 × 2 tables upon which the percentage data are based.

	Past history	Number examined	Per cent survival	% transformation among survivors	χ^2	% transformation to serotype *A*	χ^2
A. Pair 1	Standard	29	100	62	2.0	17	21.
	Starved	60	80	77		81	
Pair 2	Standard	60	95	40	14.	4	9.3
	Starved	30	97	83		42	
Pair 3	Standard	117	91	53	3.7	33	18.
	Starved	90	88	68		74	
Unpaired	Standard	357	97	45	35.	18.	23.
	Starved	120	92	77		47	
Total	Standard	563	96	47	54.7	20	71.3
	Starved	300	89	75	57.	60	73.
B. Pair 1	Standard	57	96	56	10.	16	0
	Excess food	87	99	22		16	
Pair 2	Standard	30	100	70	22.	5	0.1
	Excess food	30	100	10		0	
Pair 3	Standard	30	100	70	36.	10	0.5
	Excess food	90	100	13		42	
Pair 4	Standard	30	100	53	17.	0	0
	Excess food	90	99	16		0	
Pair 5	Standard	30	100	43	35.	23	0.6
	Excess food	90	99	2		0	
Total	Standard	177	99	58	120.	11	1.2
	Excess food	387	99	13	120.	16	0.9

observed differences in these frequencies be due merely to selective killing? If the most damaging assumptions are made about the potential serotype of killed cells, the differences between standard and starved populations remain significant. (The chi-squares for pooled data become 24 for frequency of transformation and 33 for relative frequency of transformation to *A*.) Although

these two populations, thus, do appear to differ in transforming tendency, one kind of selective killing may have occurred. The tendency in starved populations for low transformation frequency and high relative transformation to *A* to be correlated with high mortality suggests that potential serotype *B* were being selectively eliminated. Selective killing is obviously not a major determinant of the differences between standard and excess food populations.

In Table I, attention was confined to pretreatments of specific duration. What is the situation beyond these limits? If starvation is prolonged beyond three days, mortality following antiserum exposure rapidly becomes excessive; if excess feeding is prolonged beyond three days, further complications arise which will be dealt with in the following section. An extensive series of experiments dealing with animals which had been starved for periods of less than two days or kept in the presence of excess food for less than one day has not been included. The data obtained were informative only in that they failed to reveal any abrupt transition from one transformation pattern to another.

The information in Table I is concerned exclusively with tests performed upon clones 48 hours after antiserum exposure. To what extent are the differences in transforming potential a function of the arbitrary time of testing? Only 17 per cent transformation was observed after one division of 60 antiserum treated individuals with a past history of growth in excess food, and limited further subculture of 30 of the non-transformed clones revealed no later transformation. Thus, at no time after the first division, does the transformation pattern of excess food populations approximate the pattern of either standard or starved populations at 48 hours. However, when the progeny of antiserum treated animals with past histories of standard growth and starvation were examined shortly after the first division, patterns of transformation were revealed which were much less dissimilar than those observed at 48 hours. In each instance, overall transformation was enhanced and transformation to *A* depressed. The suggested complex transformations were verified by sequential testing of single clones (Table II).

A basic two-step transformation appears to be common to both populations: *D* to *B* to *A* or *D*. It is possible that both steps have occurred prior to the first division in those clones found to be either *D* or *A* at that time, i.e. that quantity and kind of *initial* transformation is the same for both populations. Additional data, not included here, do suggest that at least some two-step transformations are completed at this early stage. Regardless of the answer to this question, it is clear that most of the observable difference in transformation pattern between standard and starved populations is not a function of

TABLE II

Inconsistency of clone composition during the 48 hours following antiserum exposure of the parental animal. The first division occurred approximately 12 hours after exposure. The designation *D,B* refers to clones containing both of these serotypes.

Past history	Serotype of one product of first division	Serotype composition of sibling subclone at ca. 48 hours:							
		D	*D,B*	*B*	*B,A*	*A*	*A,D*	Other	Total
Standard feeding conditions	*D*	45	1	1	0	0	1	1	49
	B	50	5	38	3	15	2	1	114
	A	0	0	0	0	2	0	0	2
	Other	0	0	0	0	0	0	1	1
	Total	95	6	39	3	17	3	3	166
Starvation	*D*	20	0	0	0	3	0	0	23
	B	18	7	23	4	38	0	1	91
	A	0	0	0	0	17	0	1	18
	Total	38	7	23	4	58	0	2	132

initial change, but rather of either direction or time of secondary change. Probably both of these factors are important. It was never possible to obtain a stable culture of serotype *B* from these, or any other, transformations. Thus, even those clones containing only *B* at 48 hours were destined to undergo eventual secondary transformation. Transformation to both *D* and *A* occurs at this time, but no quantitative data are available on the relative frequencies of these two secondary transformations after 48 hours. If time of secondary change were the only important factor, then both populations should exhibit the same final frequency of transformation, all of it to *A*. The differences between standard and starved groups in Table I are sufficiently great that to realize this expectation most of the late secondary transformations in standard populations would have to be to *A*, most of those in starved populations to *D*. These are counter to the tendencies observed prior to 48 hours in Table II. The populations dealt with in Table II would remain distinct, in fact, on the most extreme assumptions concerning the fate of *B* animals.

Two interesting points emerge from these observations. First, as well-fed serotype *D* animals are starved, they pass through three states, as reflected by the following idealized antiserum-induced transformation patterns: *D* to *D*, *D* to *B* to *D*, *D* to *B* to *A*. These states presumably represent diverse internal environments. A plausible, though not necessary, inference is that some *D*-

determining substance is progressively depleted as a consequence of starvation. A correlary of this interpretation is that the *D*-determining substance, presumably subsurface, is inhibitory of other antigen forming processes.

The second point is superficially a paradox. In all states, primary transformation is to *B*, a serotype *not ordinarily stable* under these conditions and which readily undergoes a secondary transformation to *A* or *D*, which are stable. That the *B* antigen should be formed first, rather than *A* for example, can be explained in two general ways. First, it may be due to the prior dominance of the *D* antigen; *D* may exert a greater inhibition upon *A* than upon *B*, or it is conceivable that the syntheses of *D* and *B* antigens are related. This type of explanation cannot be excluded at present, although the frequency of similar two-step transformations involving other serotypes in stock 47 [7] and elsewhere [13] argue against it. The second type of explanation would attribute the unexpected formation of *B* antigen to the peculiar physiological conditions brought about by antiserum induced immobilization. It has been clear since the early experiments of Rössle [6] that antiserum immobilization entails a rather severe shock from which animals recover only very slowly. The effects may be manifold; for example, if antiserum is applied during a critical period preceding fission, the following division is usually abortive (unpublished data). Under the conditions of exposure employed in these experiments, recovery of motility is not complete for several hours and fission rate during the first day is appreciably retarded. It is possible that, under these transiently abnormal conditions, *B*-antigen-forming processes are favored.

An appreciation of the possible role of general physiological shock in determining the direction of antiserum induced transformations leads to the suspicion that it may play a prominent, if not predominant, role in the initiation of transformation. Direct interference with growth rate is known to effect serotype instability [9], and most other known transforming agents (proteolytic enzymes, pH, temperature, ultraviolet) also disrupt growth. Further, spontaneous transformations of serotype *D*, stock 47, appear to occur more frequently during the natural disruption of growth at autogamy [7], as was also shown by Sonneborn for variety 1 [8] and earlier by Jollos [3].

If the transient retardation of growth brought about by antiserum treatment is sufficient to bring about transformation, it is obviously unnecessary to postulate that transformation is due to the removal of inhibitors. Exposure to dilute antiserum has been shown to stimulate transformation without clearly affecting growth rate in some instances [1]. Here, some other antiserum action must be involved, possibly though not necessarily inhibitor removal.

Table III

Frequency of transformation of serotype *J* following exposure to anti-*J*-serum (*J*), anti-*D*-serum (*D*), and Ringer's solution (*R*). Animals had been grown at 27°C prior to treatment; temperature was varied during and after treatment. Almost all transformation was to serotype *D*.

Experiment	Temperature	Treatment	Number treated	Number survived	Modal number of fissions by:		% transformed clones by:	
					24 hr.	48 hr.	24 hr.	48 hr.
1	32°C	*J*	30	28	4	9	14	25
		R	30	29	5	10	32	62
	27°C	*J*	30	27	3	8	4	4
		R	30	29	4	8	0	28
2	27°C	*J*	60	52	3	7	—	19
		D	30	30	4	8	—	97

A critical test of the role of inhibitor removal is difficult to devise; experiments involving the antiserum exposure of the unstable serotypes *B*, *E*, and *J* are highly suggestive, however. It was never possible to obtain stable cultures of these serotypes and antiserum exposure did not increase the already high rate of transformation. Most significantly, the rate of transformation of serotype *J* was actually reduced by exposure to corresponding antiserum. The data from two experiments are summarized in Table III. A similar stabilizing effect of antiserum has subsequently been observed for other variety 4 serotypes [13]. In these instances, at least, inhibitor removal must be of negligible importance.

If antiserum does not induce transformation by removing inhibitors, the flux-equilibrium model is not invalidated *in toto*. As Lederberg has pointed out [4] such a system of mutual inhibitions is sufficiently flexible to account for any fallible self-perpetuating mechanism.

Remote past history and transformation pattern.—From a culture of serotype *D* which had been maintained under standard feeding conditions for over 20 days, eight subcultures were initiated at different times and maintained in the presence of excess food. All of these subcultures eventually underwent massive transformation to serotype *J*, between 20 and 30 days after initiation. Due, presumably, to the instability of *J*, the *J*:*D* ratio never exceeded about 0.5. Shortly before the spontaneous appearance of *J* cells in these populations, it could be shown that, when treated with antiserum, the *D* cells transformed with a high frequency and to *J*. Sequential tests on clones

obtained from antiserum exposed animals suggested that this was the primary transformation and that, although the *J* were extremely unstable, they reverted almost exclusively to *D*.

In the preceding section, we dealt with quantitative variations of one basic pattern of antiserum induced transformation: *D* to *B* to *A* or *D*. By prolonged growth in excess food, a qualitatively different potentiality is obtained, expressed by the antiserum induced pattern of transformation: *D* to *J* to *D*. These two qualitatively different states will be referred to as *D*(*B*) and *D*(*J*) respectively.

In order to determine the duration of growth in excess food required to bring about the shift from *D*(*B*) to *D*(*J*), daily subcultures were made from a standard population and maintained in excess food. At intervals, samples of these subcultures were removed, exposed to antiserum, and transformation at 48 hours examined. In this experiment (Table IV A), antiserum induced transformation was predominantly to *B* at least until the fourth day in the presence of excess food; transformation to *J* did not assume importance until about the twentieth day. Serotype *J* began to appear spontaneously in the population at about 25 days, and was not observed following control treatment with Ringer's solution before this time. The inertia of this system in responding to a potentially effective environmental change is not obviously compatible with a flux-equilibrium model, nor for that matter with any existing model.

The possibility that mutant selection is responsible for the delay seems unlikely in view of the ability of *D*(*J*) cells to return to the *D*(*B*) state without appreciable cell-division when starved (Table IV B). The possibility that the pertinent environmental factor is, in fact, being altered only very slowly is not excluded, but the following points argue against it. First, *D*(*J*) populations are not quickly converted to *D*(*B*) populations by a return to standard conditions of growth (Table IV C). Second, three daily isolation lines of *D*(*B*), grown in excess food and washed before each isolation, eventually became *D*(*J*) but showed no alteration of antiserum-inducible transforming pattern for at least 30 cell generations.

Another possibility, which was not considered at the time the experiments were performed, is formally consistent with the facts. This involves the assumption that a given macronucleus is limited in the array of serotypes it may determine, in much the same way it is limited as to mating-type determination. This limitation might be either relative or absolute. A shift, then, in basic transforming potential, such as from *D*(*B*) to *D*(*J*), could not occur except at the time of a nuclear reorganization (autogamy) which, in excess

TABLE IV

Effects of prolonged culture under different feeding conditions upon the pattern of transformation of serotype *D* following antiserum exposure. Transformants which were not serotype *J* were almost exclusively serotypes *A* or *B*.

Past history	Number examined	Per cent survival	% tranformation among survivors	% of transformation to serotype *J*
A. Standard conditions for 20 days, then excess food for:				
0 days	180	100	57	3
1	180	98	15	6
2	150	100	16	0
3	120	100	12	8
4	120	100	14	17
5–6	120	99	2	100
7–9	150	100	11	100
11–15	90	100	9	—
20	60	100	43	100
30	30	100	57	100
B. Excess food for 20 days, then no food for:				
1 day	47	98	78	89
2	18	83	93	93
3	21	100	86	6
4	24	96	91	10
5	76	96	86	3
6	19	100	95	6
7	17	100	82	0
8	81	98	78	2
C. Excess food for 20 days, then standard conditions for:				
1 day	60	100	65	100
3	120	93	21	75
6	60	95	96	67
20	60	95	40	0

food, might entail a considerable delay. Since growth is arrested at autogamy, this explanation has the additional virtue that it could account for the apparently inconsistent observations that, on the one hand, *D*(*J*) and *J* arise from *D*(*B*) under conditions conducive for rapid growth, whereas *J* is stabilized by antiserum treatment which retards growth.

SUMMARY

The effects of past feeding conditions upon the antiserum induced transformation pattern of serotype *D*, stock 47, variety 4, *Paramecium aurelia*, were investigated. Remote past history was found to affect the direction of initial transformation. After prolonged growth under conditions of limited food, animals respond to antiserum exposure by transforming to serotype *B*. After prolonged growth under conditions of excess food, they respond to the same exposure by transforming to serotype *J*. The inertia of the response to environmental change is not readily explained; it is suggested that this may mean that shifts in basic transforming potentiality can occur only at autogamy.

The immediate past history of animals which tend to transform to *B* was found to affect the frequency of this primary transformation and also the relative frequency with which the derived *B* undergo secondary transformation to serotype *D* or serotype *A*. The frequency of primary transformation and the frequency of transformation to *A* increase with starvation.

The fact that there is an unstable *B* intermediate in the transformation of *D* to *A*, together with the fact that antiserum may depress rather than increase the transformation of certain unstable serotypes (e.g. serotype *J*), suggest that inhibitor removal may not be the important means by which antiserum induces transformations. It is proposed instead that transformation may be due to the generalized physiological shock accompanying immobilization by antiserum.

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A METHOD TO IMPROVE CONTRAST IN HIGH RESOLUTION ELECTRON MICROSCOPY OF ULTRATHIN TISSUE SECTIONS[1]

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The routine resolving power of a well-trimmed modern electron microscope is better than 20 Å. For the new Siemens electron microscope a resolving power better than 10 Å has been clearly demonstrated. The best specimen resolution that we have been able to obtain in our laboratory on ultrathin tissue sections has been about 25 Å. This resolution was obtained in 1952 [2–7], but in spite of an improvement of our sectioning technique [8] this maximum resolution has not been increased.

When analyzing the reason for this limitation it could be excluded that the specimen resolution reflected the actual resolving power of our instrument. The resolving power of our instrument has routinely been kept at 20 Å or better when high resolution pictures were required. The specimen resolution was found to be highly influenced by a general granularity covering the whole image area. This granularity is due to a phase-contrast effect which was clearly demonstrated when examining the pictures of through-focus series (Fig. 1). The granularity became finer the closer to focus the picture was taken and was not present in the in-focus pictures where only the graininess of the photographic emulsion interfered. In slightly underfocussed pictures as in Fig. 1 B the average distance between the individual "granules" was 15–20 Å. This distance became increased to 30–40 Å with slightly increased underfocussing. This phase-contrast effect is due to disturbances of the wave front when passing through the specimen. The granular structure appeared identical all over the image surface, over areas covered by tissue sections as well as areas of uncovered formvar film. Therefore, it appears as rather unspecific and is unrelated to the structure of the tissue itself. This phase-contrast effect might be due to minute inhomogeneities of the formvar film and of the contamination layer built up on the specimen during its exposure to the electron beam.

[1] This paper is based on a communication given at the Scandinavian Electron Microscope Society meeting in Stockholm, May 13, 1955.

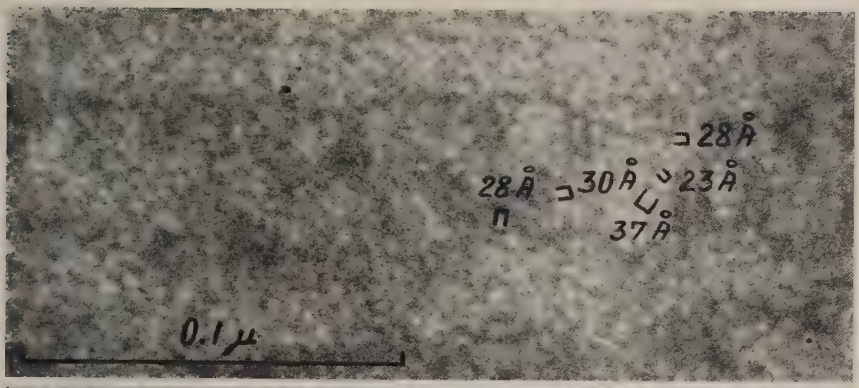
The contrast conditions of this background granularity are such that they will seriously interfere with low contrast specimens, for instance ultrathin tissue sections. They will in fact have a similar effect on the resolution of the picture as has the screen in printing on the details of the printed picture. The fact that this phase-contrast effect is absent in in-focus pictures is of no avail as the contrast conditions of ultrathin tissue sections mounted in the conventional way on a formvar film unfortunately are such that a certain degree of underfocussing is necessary to secure sufficient contrast. Therefore, the key problem would be to increase the contrast of our specimens to render it possible to make use of close-to-focus or in-focus pictures.

The ultrathin tissue sections are conventionally mounted on a formvar film in order to stand the electron bombardment in the microscope. The thickness of this formvar film is about 200 Å. As the thickness of the thinnest ultrathin sections that we are using is less than 100 Å, this layer of formvar contributes proportionally to a very great extent with diffusely scattered electrons which reduce the image contrast.

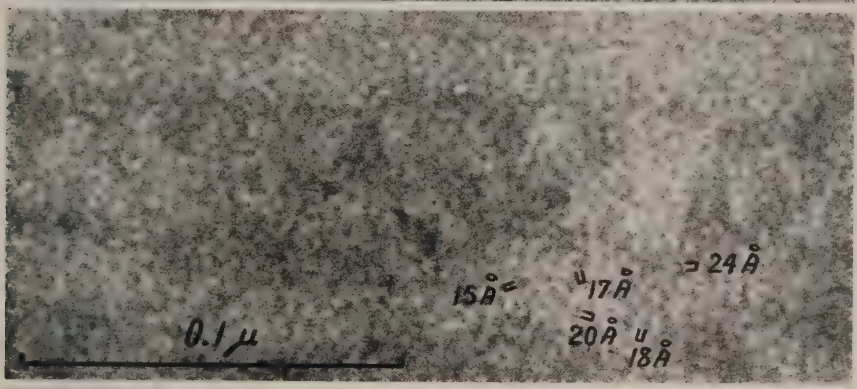
It therefore appears important for an improvement of the contrast to reduce this scattering, for instance most radically through mounting the ultrathin sections without a formvar film. This seemed possible, for it was occasionally observed that the sections would not disrupt in the electron beam when extending over holes in the supporting film. It was obvious that the image contrast in such regions was very much higher than in areas with formvar film. A method was then worked out to prepare a supporting structure with the shape of a net with minute holes and very narrow mesh to replace the formvar films.

Such supporting nets may be prepared in the following way. A microscope slide with surface etched with hydrofluoric acid is dipped into a 0.2–0.3 per cent formvar solution in ethylene dichloride. Immediately when lifting the slide out of the formvar solution and before the ethylene dichloride has evaporated a light breathing on the slide will make water vapor condense onto its surface to form minute water droplets in the thin layer of formvar solution.

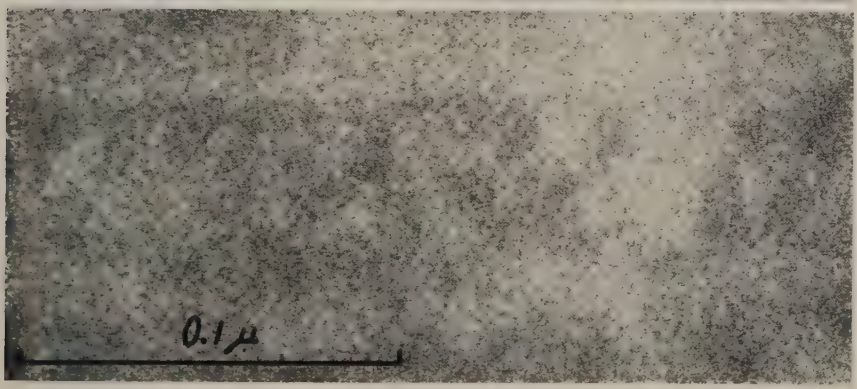
Fig. 1. High power electron micrographs of ultrathin tissue sections showing three pictures in a through-focus series. The underfocussed Figs. A and B show the general background granularity that is due to the phase contrast effects. In Fig. A the shortest distances between the individual granules are 20–40 Å. In the less underfocussed Fig. B the similar granularity is seen with the shortest distances between the granules being 15–20 Å. Fig. C is an in-focus picture showing a minimum of phase contrast effects. The graininess of the photographic emulsion is shown in this picture as well as the reduced contrast. Magnification 560,000 ×. (Electron optical magnification 25.000 ×.)



A



B



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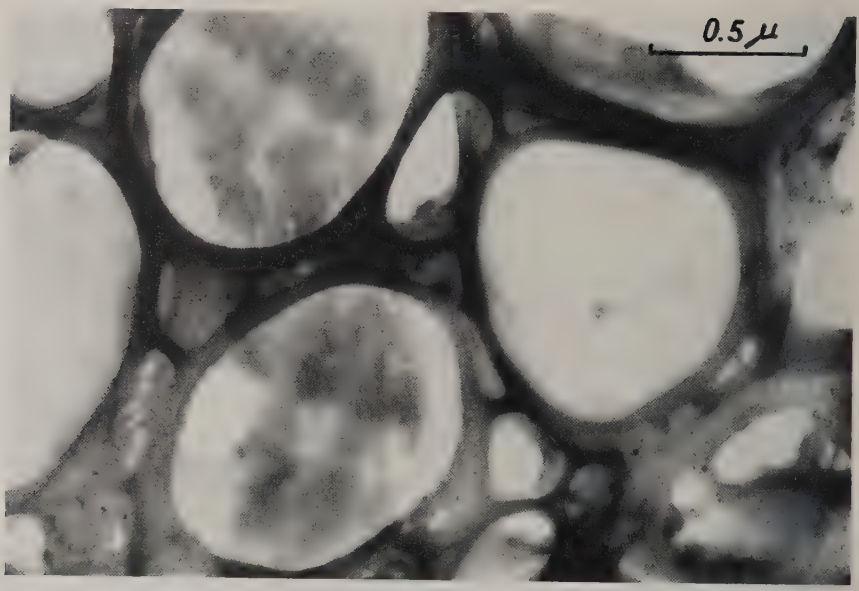

Fig. 2. Survey picture showing the ultrathin tissue section mounted on a metallized formvar net as support instead of a formvar film. Part of a myelin sheath of a nerve fiber in the sciatic nerve of the mouse is seen extending over two of the holes. Magnification 46,000 ×.

When the ethylene dichloride and the water evaporates, the formvar film formed contains densely arranged air bubbles.

If such a film is floated off onto a water surface from an ordinary slide that has not been etched, rater few holes will be present. At the sites of the air bubbles the formvar film is very thin. If, however, the film is floated off onto water from an etched glass surface these thin parts of the film will rupture and holes are formed.

The size of these holes is then adjusted through exposing the formvar nets mounted on regular copper grids to the electron beam in the microsope. By adjusting the intensity and the exposure time, almost any desired hole size may be obtained. The diameter of the holes of the formvar nets that we have been using is 0.5–1μ.

Fig. 3 (right). High magnification of part of a section extending over a hole in a supporting net. Part of the rim of the hole is seen and part of the myelin sheath of a nerve fiber in the sciatic nerve of the mouse extends over the hole. Notice the very close-to-focus picture. Magnification 100,000 ×.

0.1μ

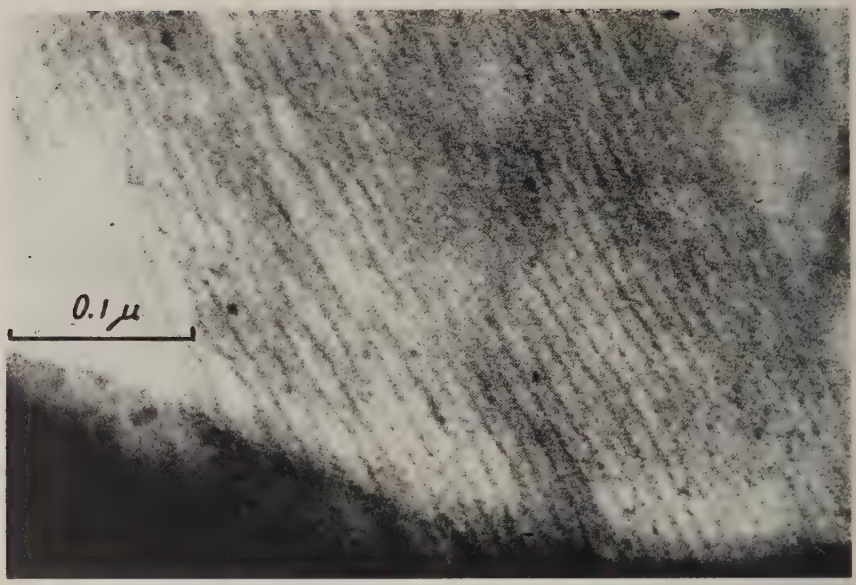

Fig. 4. Higher magnification of a region of a part of Fig. 3 showing the regular laminated structure of the myelin sheath at a very high contrast in spite of this picture being taken very close to or in focus. Magnification 270,000 ×.

To stabilize these formvar nets they are enforced through evaporation of metal such as aluminium, or of carbon.

The tissue sections are mounted on the copper grids covered with the metalized formvar net in the regular way. Fig. 2 shows a part of such a net with an ultrathin cross section of the sciatic nerve of mouse mounted. The myelin sheath of a cross section of a nerve fiber extends over two of the holes.

When examined and photographed in the electron microscope these specimens are astonishingly stable. Thermal drift interference is not appreciably greater when working with these specimens than with the regular specimens of sections mounted on formvar films. The focussing is very much facilitated through the presence of the sharp rims of the holes.

The most striking gain is the tremendous increase in contrast which makes it possible to work with in-focus pictures and still have sufficient contrast. Figs. 3 and 4 show high magnifications of an in-focus picture of the myelin sheath of a cross-sectioned nerve fiber in the sciatic nerve of the mouse.

The limiting factor which now has to be seriously considered is the graininess of the photographic emulsion. The resolving power of the photographic emulsion differs very much when dealing with high contrast pictures as compared to low contrast pictures. In the latter case it is much less than that estimated under optimum conditions on a high contrast picture as are the figures presented by the manufacturers of photographic plates and films. In low contrast pictures the resolving power of the photographic emulsion will depend on rather course groups of grains as structural elements of the picture and not on the size of the individual grains.

When estimating the resolution of our electron micrographs we demand that image points which represent the shortest distance that is resolved should be resolved on two or more different pictures of the same field [1]. When these rigorous conditions are applied there is clear evidence how easily groups of silver grains may be misinterpreted as image details. In fact, we have not been able to separate and identify any image points on the photographic plate, which are closer than a distance of 50–60 μ between their centers. With an electron optical magnification of 25,000 times this means an optimum resolution not better than about 20 Å due to the graininess of the photographic emulsion. The resolving power of the emulsion does not differ very much when comparing different types of emulsions and testing them at low contrast conditions [E. Ruska, personal communication]. Especially fine grain emulsions do not represent any very marked advantage [E. Ruska, personal communication].

The improvement of the contrast obtained through the mounting of ultrathin tissue sections (thickness less than 100 Å) means that the conditions for top resolution on these low contrast specimens are improved. It should now be possible to make use of the top resolution of less than 10 Å when working on tissue sections. These possibilities are now being explored.

SUMMARY

The general background granularity observed in out-of-focus electron micrographs is discussed as a phase contrast effect, that prevents higher resolution than about 25 Å on low contrast specimens as ultrathin tissue sections. The problem of improving the specimen resolution when dealing with ultrathin sections consists of improving the contrast so that in-focus pictures may be used. A method of mounting the sections on formvar nets is described, which makes it possible to obtain pictures of sections, which

are partially not supported by a formvar film. The reduced diffuse scattering of the electrons when passing through these areas means a drastic increase of contrast.

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RELATIONSHIP OF CATALASE AND SODIUM NITRITE TO PROTECTION AGAINST X-RAYS[1]

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Chemical protection against ionizing radiations involves so many variables in the metabolizing cell that it is often difficult to isolate the precise mechanism involved; it is, in fact, often difficult to eliminate even one of several possible mechanisms. Add to this the interplay of complex organ systems, and one must agree with Patt [14] that the mechanisms of chemical protection in higher organisms are poorly understood; protective substances may increase the resistance to X-rays by modifying either activated water reactions, the nature of biological targets, the redox equilibrium of the cell, or the cellular enzymes.

Sodium nitrite has attracted considerable interest in the complex problem of radiation protection, but the results obtained by its use have been equivocal. Where protection has been reported [15, 13, 9] it has been attributed to various mechanisms, one of which, enhancement of catalase activity, may be tested in a straightforward, *in vitro* experiment involving one of the simplest of biological entities, bacteriophage T1. In spite of its comparative simplicity, this phage responds to a wide range of protective agents paralleling those commonly employed in radiation protection in higher forms of life. T1 has the distinct advantage over other biological material in that it can be readily transferred to solutions of highly varied osmotic concentration [2].

Since sodium nitrite has been shown to protect bacteriophage T1 against X-rays [4], the present study was designed to test the hypothesis of enhancement of catalase activity, by the addition of sodium nitrite and/or catalase in an aqueous medium to this non-metabolizing bacteriophage, which is less likely to obscure elementary radiation processes than more complex biological systems.

The basic assumption in the hypothesis of enhancement of catalase activity by sodium nitrite is based on the work of Chance [8, 5] who has shown that the reaction of catalase with hydrogen peroxide involves the formation

[1] This investigation represents a part of the research performed under Contract No. AT(11-1)-205 between the Atomic Energy Commission and the University of Notre Dame.

of a catalase–H_2O_2 complex. Once formed, this complex does not decompose spontaneously under ordinary circumstances, but is relatively stable unless there occurs collision and subsequent reaction with a donor molecule, such as *sodium nitrite*, ethanol, methanol, sodium formate, or another H_2O_2 molecule.

EXPERIMENTAL PROCEDURE

Bacteriophage.—The methods of cultivation of the test organism, bacteriophage T1, specific for *Escherichia coli*, strain B, were essentially those outlined by Adams [1]. The virus stock consisted of the filtered, cell-free lysate of T1, incubated 24 hours with *E. coli* in a chemically defined medium, adjusted to pH 6.8, and consisting of the following chemicals per liter: NH_4Cl, 1.0 g; $MgSO_4$, 0.1 g; KH_2PO_4, 1.5 g; Na_2HPO_4, 3.5 g; lactic acid, 9.0 g. This stock, consisting of 2×10^{10} particles per ml, was first diluted 1:100 in distilled water and then 1:100 in the various solutions, giving working stocks of 2×10^6 particles per ml for both irradiated samples and controls. In the tests conducted, no discernible effects could be attributed to the chemical composition of the original lysate which had been diluted 10,000-fold during the experimental treatment.

Chemicals.—Water doubly distilled in pyrex glass was used in all solutions and media. Solutions were made anew before each radiation experiment and agitated on a mechanical stirrer in order to be brought into equilibrium with air at room temperature. $NaNO_2$, C.P., from Fisher Scientific Co. was used for the nitrite solutions. Crystalline catalase was procured from General Biochemicals, Inc. and from Worthington Biochemical Corp. Catalase activity tests followed essentially the titrimetric method originally described by von Euler and Josephson [12]. *Katalasefähigkeit* (*Kat.f.*) values averaged approximately 14,000.

Irradiation.—The X-ray tube with tungsten target was operated at 100 kv and 8 ma with a 1 mm beryllium window serving as the only filtration. Samples of 1 ml, each dispensed in open, flat-bottom vials 2.7 cm in diameter, were exposed to the X-ray beam in a temperature-controlled device at 25°C. Effects of temperature during X-irradiation have been found to be considerable, particularly on phage in the more dilute solutions. The dose delivered at the center of the irradiated suspension was 12,500 r/min.; the suspension was 1.8 mm deep. In order to prevent post-irradiation effects, which are very pronounced in dilute salt and catalase solutions, Difco nutrient broth in 3×10^{-2} concentration was added within 10 seconds after termination of irradiation. The diluting and plating processes followed immediately. Survival values represent the means of six experiments.

RESULTS

In order to determine the extent to which catalase destruction by irradiation might be operative under the conditions of these experiments, a series of catalase solutions, varying in concentration from 10^{-7} to 10^{-3} was divided

into two lots, one kept as a control and the other irradiated with 45,000 r. Activity tests in duplicate were run on the samples, and the ratios of the activities after irradiation to the activities before irradiation are plotted in Fig. 1. The results are in substantial agreement with those of Tytell and Kersten [16] and show that in the range within which most of the present experimentation was carried out, the average decrease in catalase activity

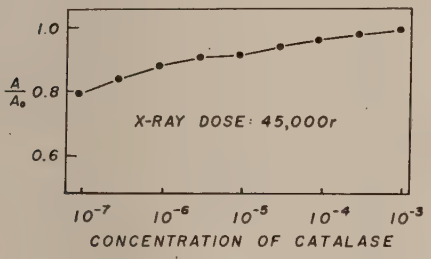
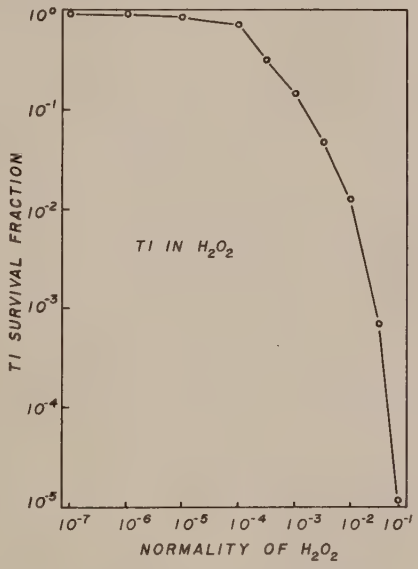


Fig. 1 (left). Relative catalase activity after irradiation, as a function of concentration of catalase. A/A_0 = ratio of catalase activity before and after 45,000 r of X-rays. Concentrations are in terms of grams of crystalline catalase per milliliter of phosphate buffer.

Fig. 2 (right). Survival of bacteriophage T1 after 15 minutes in hydrogen peroxide, as a function of normality of hydrogen peroxide.



as a result of irradiation did not exceed 10–12 per cent. Fig. 2 shows the susceptibility of T1 to H_2O_2 in $M/150$ phosphate buffer, adjusted to pH 6.80. The ability of catalase to protect T1 against X-ray inactivation has been established with four different lots of crystalline catalase. The protective ability varies somewhat from sample to sample, depending on the degree of purity of the catalase.

Catalase has been shown to exercise considerable protection in virtue of its specific enzymatic activity [3]. Fig. 3 shows the survival fraction of T1 after irradiation in various concentrations of sodium nitrite and catalase. In Fig. 3 when the survival fraction of irradiated phage is plotted against the concentration of protective agents, each on a logarithmic scale, it can be seen that there is a range within which the curve is essentially linear for both sodium nitrite and catalase. It is necessary in protection studies to keep

the concentration well within this range; otherwise, at higher concentrations, the relative efficiency of the protective agent is reduced and a true evaluation of its protective ability cannot be made. At lower concentrations, on the other hand, the protective ability of all reagents approaches values characteristic of distilled water.

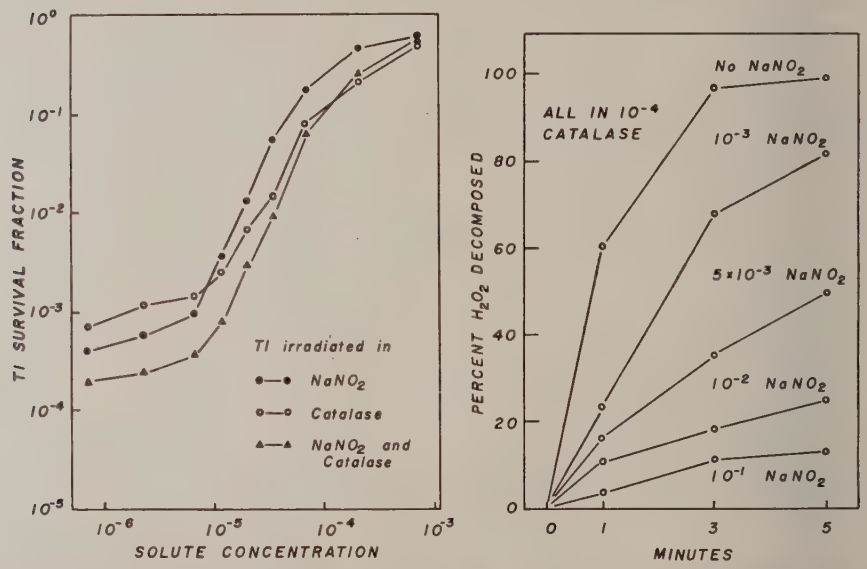


Fig. 3 (left). Survival of bacteriophage T1 after 37,500 r of X-rays, as a function of concentration of catalase and sodium nitrite and of mixtures of catalase and sodium nitrite. Concentrations are on weight basis, and the concentration of the mixture represents the total weight of solute.
Fig. 4 (right). Percentage of hydrogen peroxide decomposed, as a function of time, with catalase only, and with catalase and increasing concentrations of sodium nitrite.

A superficial inspection of Fig. 3 indicates that mixtures of catalase and nitrite are less efficient protective agents when present together than when present separately. It should be noted, however, that the lower line in Fig. 3 represents the *total* amount of solute present; the amount of catalase or nitrite therefore is only half of the total concentration and interpretations must be made accordingly.

There is a problem in determining whether one reagent enhances the activity of another when each contributes separately to the protective pattern. If one reagent, which alone did not affect the survival of irradiated T1, could

be added in small amounts to a protective agent, it would be possible to discern enhancement of the other protector merely by checking the resultant survival to ascertain whether it had been increased. This simple method cannot be applied in determining if one agent is potentiating the action of another agent when both are protective and acting simultaneously. The present evaluation of the protective ability of two agents is based on a modification of analyses previously used in other protective studies [4, 11].

The survival of T1 after X-irradiation was determined in a number of solutions, as follows:

1. The concentrations of nitrite and catalase were first used separately, and then the solutions were mixed. The resultant total concentration of solute was the same, whereas the separate concentrations were reduced one-half. The results are shown in Table I.

2. The concentrations of nitrite and catalase were used separately and mixtures of the two were made up so that the concentrations of the two com-

TABLE I

Protective abilities of sodium nitrite and catalase for bacteriophage T1.

P = gross protective ability = $D_1\ D_2$, in which D_1 = dose in roentgens required to give 37 per cent survival in presence of designated solute; D_2 = dose in roentgens required to give 37 per cent survival in distilled water. p = specific protective ability = $P/\mu g$, in which μg = weight of protector in micrograms per milliliter. p_{c+n} = specific protective ability of mixtures of catalase and sodium nitrite.

Concentration of solute	Phage survival (N/N_0)	P	μg/ml of solute	p	p_{c+n} (Expected)*	p_{c+n} (Experimental)
7×10^{-4} $NaNO_2$ . . .	6.1×10^{-1}	71,730	700	102	90	
7×10^{-4} Catalase . . .	5.3×10^{-1}	54,930	700	78		
7×10^{-4} $NaNO_2$ + Cat. .	5.6×10^{-1}	60,630	700	87		87
7×10^{-5} $NaNO_2$ . . .	1.8×10^{-1}	17,830	70	255	202	
7×10^{-5} Catalase . . .	7.5×10^{-2}	10,430	70	149		
7×10^{-5} $NaNO_2$ + Cat. .	6.3×10^{-2}	9,530	70	136		136
2×10^{-5} $NaNO_2$ . . .	1.4×10^{-2}	4,710	20	236	204	
2×10^{-5} Catalase . . .	6.8×10^{-3}	3,430	20	172		
2×10^{-5} $NaNO_2$ + Cat. .	3.0×10^{-3}	2,400	20	120		120
7×10^{-6} $NaNO_2$ . . .	9.7×10^{-4}	1,340	7	192	218	
7×10^{-6} Catalase . . .	1.5×10^{-3}	1,700	7	243		
7×10^{-6} $NaNO_2$ + Cat. .	3.7×10^{-4}	680	7	97		97

* Mean value expected if the two components act independently without mutual inhibition or enhancement of protective abilities.

ponents remained unchanged, whereas the resultant total concentration of solute was doubled. The results are shown in Table II.

TABLE II

Protective abilities of sodium nitrite and catalase for bacteriophage T1.

(P = gross protective ability and p = specific protective ability, as in Table I.)

Concentration of solute	Phage survival (N/N_0)	P	μg/ml of solute	p	p_{c+n} (Expected)*	p_{c+n} (Experimental)
3.5×10^{-4} $NaNO_2$ . . .	5.3×10^{-1}	55,030	350	157		
3.5×10^{-4} Catalase . .	3.5×10^{-1}	31,680	350	91	124	
7.0×10^{-4} $NaNO_2$ + Cat.	5.6×10^{-1}	60,630	700	87		87
3.5×10^{-5} $NaNO_2$ . . .	5.5×10^{-2}	8,880	35	254		
3.5×10^{-5} Catalase . .	2.5×10^{-2}	6,100	35	174	214	
7.0×10^{-5} $NaNO_2$ + Cat.	6.3×10^{-2}	9,530	70	136		136
1.0×10^{-5} $NaNO_2$ . . .	3.8×10^{-3}	2,660	10	266		
1.0×10^{-5} Catalase . .	2.5×10^{-3}	2,190	10	219	243	
2.0×10^{-5} $NaNO_2$ + Cat.	3.0×10^{-3}	2,400	20	120		120
3.5×10^{-6} $NaNO_2$ . . .	7.2×10^{-4}	1,120	3.5	320		
3.5×10^{-6} Catalase . .	1.3×10^{-3}	1,610	3.5	460	390	
7.0×10^{-6} $NaNO_2$ + Cat.	3.7×10^{-4}	680	7.0	97		97

* As in Table I.

From the protective values listed in Tables I and II the following may be deduced:

1. High concentrations of protector give greater overall protection than lower concentrations, as indicated by the values of P (gross protection).

2. The protective ability per unit weight of protective agent is low in the high concentrations, but increases in intermediate concentrations. The protective ability per unit weight of protective agent is low in the high concentrations, but increases in intermediate concentrations. This is indicated by the values of p (specific protection).

3. Protective ability of the two protectors present simultaneously is in all cases lower than would be expected if the two components were acting independently without mutual inhibition or enhancement of protective abilities.

The decreased protective ability of catalase in the presence of nitrite is reflected in the direct titrimetric assays designed to compare the ability of catalase alone to decompose H_2O_2, and catalase in the presence of various

concentrations of $NaNO_2$. Fig. 4 shows one set of typical values. Similar results were secured with other concentrations of catalase and varied concentrations of $NaNO_2$. The results indicate that the ability of catalase to decompose H_2O_2, based on reaction rate, was impaired by the presence of $NaNO_2$ rather than enhanced.

DISCUSSION

The results here presented indicate clearly that sodium nitrite and catalase each serve as efficient protective agents for bacteriophage T1 against X-irradiation. Together they are somewhat less efficient than when each acts independently; in other words, when acting together sodium nitrite fails to potentiate the activity of catalase; moreover, the results fail to show simple additivity.

Interest in the mechanism of protection is heightened by the contributions of Chance [5] who states that catalases are apparently specific for a particular functional group of the substrate and donor molecule. The peroxide group, –OOH, of the substrate is essential; disubstituted peroxides are inactive. The donor molecule may be a secondary alcoholic group, a nitrite, or a hydrogen peroxide molecule, HOOH. The addition of a suitable donor such as formate or nitrite causes catalase to act *peroxidatically*, as opposed to *catalatically* when hydrogen peroxide is the donor; each reduces the steady-state peroxide concentration, the latter more efficiently. Chance estimates that about four times as much peroxide is decomposed catalatically as peroxidatically in the respiring cell. The presence of an excess of donor, such as nitrite, permits the reaction to proceed utilizing sodium nitrite and hydrogen peroxide, whereas in the absence of sodium nitrite, hydrogen peroxide becomes the donor molecule and the reaction proceeds utilizing hydrogen peroxide as both substrate and donor. In the latter case there would be a more rapid destruction of hydrogen peroxide than in the former. Since sodium nitrite causes the peroxidatic decomposition of hydrogen peroxide, proportionately less hydrogen peroxide is actually decomposed in the presence of sodium nitrite than when hydrogen peroxide is both substrate and donor.

Situations might be conjectured in which, despite the above considerations, sodium nitrite might serve to enhance protection *via* catalase. If, for example, the concentration of hydrogen peroxide were at such a critical level that it was all tied up in the enzyme–substrate complex, then barring a suitable donor, such as sodium nitrite, it would remain in this undecomposed complex. In such a situation, the sodium nitrite would be without

particular merit, unless one were to postulate that the enzyme–substrate complex itself is harmful to the organism. In this case the destruction of the complex by reaction with a donor molecule would be tantamount to affording protection to the organism. In any case, it would not be correct to say that the production of more hydrogen peroxide would *ipso facto* produce more damage to the organism even if all the catalase and hydrogen peroxide were tied up in the enzyme–substrate complex, since additional hydrogen peroxide could function as a donor and thus be eliminated efficiently.

Another case in point would be the question whether, if all the catalase were tied up in the enzyme–substrate complex, the addition of sodium nitrite as a donor might accomplish the liberation of catalase more rapidly than if hydrogen peroxide were the donor. An answer may be found in the relative magnitude of the velocity constants for the reaction of the enzyme–substrate complex with hydrogen peroxide as the donor and with nitrite as the donor. Coupled oxidations would occur as the normal result of competition between the two donor molecules, the nitrite and the peroxide molecule, for the catalase–hydrogen peroxide complex. The velocity constant for the reaction with nitrite as donor [5] should be approximately 2×10^{-4} that of the constant for hydrogen peroxide. Although the reactive species in the nitrite reaction has been found to be the free acid, for which the velocity constant is much greater [6], still, with a given total molarity of nitrite the reaction will not proceed at any faster rate than indicated above [7]. This would mean that if the nitrite concentration were approximately 5000 times as great as the hydrogen peroxide concentration, the two donor molecules would compete on a roughly equal basis. Under such conditions it would appear that nitrite would not be particularly effective in liberating catalase from the enzyme–substrate complex since the hydrogen peroxide would accomplish the same effect just as efficiently in much lower concentration.

A considerable excess of sodium nitrite (5000 times that of hydrogen peroxide) would enable the nitrite to compete as donor with the hydrogen peroxide on an approximately equal basis. If, however, the concentration of nitrite in the biological system were kept within reasonable limits, say 10^{-4} to 10^{-3}, the correspondingly lower concentration of hydrogen peroxide would probably not affect the biological system, at least not the bacteriophage. In relatively high concentrations of nitrite relative to hydrogen peroxide, moreover, the reducing ability of the nitrite should rather efficiently take care of any hydrogen peroxide that might form in concentrations high enough to be harmful to the biological system.

On the basis of these considerations, our radiation results and our titri-

metric determinations of catalase activity are quite in harmony with the theories on the mechanism of catalase activity. The fact that nitrite alone affords very efficient protection against X-irradiation, although not by enhancement of catalase activity, remains to be explained. A possible explanation may be sought in the light of oxidation and reduction reactions in irradiated water. Dainton and Collinson [10] have considered the capacity of irradiated water to oxidize certain solutes and to reduce others, and have expressed this ability as the equivalent redox potential. Applying these concepts to irradiated biological systems, we logically expect that reducing agents may figure importantly in protection, and the available evidence for this is presented by Patt [14] in his review of protective mechanisms involved in ionizing radiation injury. The reducing ability of sodium nitrite would make it qualify as a protective agent without recourse to the catalase–nitrite donor mechanism.

Our postulate, therefore, is that sodium nitrite protects bacteriophage T1 against X-rays chiefly by acting as a reducing agent. As a donor molecule enabling the catalase–hydrogen peroxide complex to proceed to completion, it is inefficient as a protective agent. While these results are not here extrapolated to higher organisms, their significance in interpreting radiation injury is evident.

SUMMARY

The ability of sodium nitrite to protect various biological systems against X-rays has been attributed to several mechanisms, one of which, enhancement of the ability of catalase to decompose hydrogen peroxide, has been investigated with bacteriophage T1. In order to establish the validity of using catalase as a protective agent for phage against X-rays, the ability of catalase to withstand X-irradiation was determined under the conditions of these experiments, and the sensitivity of bacteriophage T1 to hydrogen peroxide was established.

The phage was then irradiated in the presence of varying concentrations of catalase and of sodium nitrite, and of the two combined. High protection was afforded the phage by high concentrations of catalase and of nitrite, with gross protection falling off rapidly when low concentrations of solutes were employed although specific protection (protection per microgram of solute) was increased. In the presence of the two protective agents combined, there was impairment rather than enhancement of the ability of catalase to protect T1 against X-rays. The impaired activity is explained by increased peroxidatic action, which proceeds at the expense of the catalatic action, of catalase.

In the catalatic reaction, hydrogen peroxide acts as both substrate and donor molecule, and the velocity constant for the reaction with hydrogen peroxide as donor is many times greater than with nitrite as donor.

Sodium nitrite does not enhance the protective ability of catalase for irradiated T1, nor does it enhance the ability of catalase to decompose hydrogen peroxide; nevertheless, nitrite alone is an efficient protector, acting presumably as a reducing agent.

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METABOLIC CHANGES IN FREE AMINO ACIDS AND PEPTIDES DURING URODELE DEVELOPMENT[1]

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That protein metabolism plays an essential role in development would seem to be self-evident. First, there is a continuous formation of differentiating tissues in various organ-systems. Second, according to the results of serological studies, synthesis of new antigens occurs in the course of development [6, 11, 27]. These processes involve the breakdown of egg proteins and the conversion of them into specific proteins of another form. Obviously, studies of the metabolic changes in free amino acids and peptides at various developmental stages provide us with valuable information for an understanding of the mechanism of such chemical differentiation.

In amphibian development efforts have been made to correlate the changes in free amino acids and peptides to morphogenetic processes. However, the early approaches to this problem by means of chromatographic techniques appeared to be negative. It was first reported that no free amino acids except small quantities of peptides could be traced in the frog embryos [24]. Later experiments showed that these substances do exist in detectable amounts in embryos of *Rana pipiens*, *Hyla regilla* and other species, but no marked changes were observed in the course of development [8, 20]. More recently Kutsky, Eakin, Berg and Kavanau [22], using the method of microbiological assay in *Rana pipiens* have succeeded in demonstrating quantitative changes of free and non-protein amino acids at stages of development ranging from the unfertilized egg to the neurula. Both chromatographic and microbiological assay studies have also shown that distinct changes in amino acids take place in sea urchin development [21]. The recent work of Deuchar [7] on *Xenopus laevis* embryos has again confirmed the fact that the free amino acid content varies according to developmental age, and differences have been detected even between dorsal and ventral halves of the embryos.

In a previous paper it has been reported that definite changes in free ninhydrin-positive substances were observed by partition paper chromato-

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graphy in the urodele development even when only a small number of embryos were used [5]. Since then this investigation has been extended by carrying out more detailed quantitative measurements on embryos of three different urodeles. The identification of several doubtful spots presented in the last paper has also been checked. Our knowledge concerning the relation between morphogenesis and protein metabolism has been based so far mainly on anuran embryos. The present report is intended to clarify this problem further by using urodeles as working material.

MATERIALS AND METHODS

This investigation was carried out largely on embryos and larvae of the alpine newt, *Triton alpestris*. In addition, two other species, *Triton palmatus* and *Triton cristatus*, were used for determinations of total free ninhydrin-positive substances. The animals were brought to the laboratory at the beginning of the spawning season, kept in running water and given constant feeding. Batches of eggs were daily collected from the container and kept at 18°C until the desired stage was reached.

The embryos were taken out of the jelly immediately before the preparation of extracts. For stages ranging from freshly fertilized eggs to late gastrulae, the vitelline membrane was not removed in order to avoid the loss of material by operation. Preliminary tests had shown that this membrane contained no detectable amino acids. However, it could easily be taken off without injuring the embryo from the early neurula stage.

During sampling the developmental age of individual embryos was examined according to the table of development originally prepared by Harrison for *Amblystoma punctatum*. (The early stages of this table have been cited by Lehmann [22] and a complete series of sketches presented by Hamburger [18] and Rugh [28].) Only those individuals which showed normal development and reached the same developmental stage were used for each determination. The investigation included stages ranging from the first cleavage to newly hatched larvae. Even at the latest stage studied the gut of the larva still contained much yolk and there was no intake of food.

The paper chromatography technique used in the present experiment was essentially the same as that published by Hadorn and Stumm-Zollinger [17] in their study on *Drosophila* lethal mutant. For assaying the total free amino acids and peptides one-dimensional chromatography was used. For each determination two freshly prepared embryos were placed at the starting point of the filter paper (Whatman No. 1) and squashed slightly with a thin glass rod. The chromatogram was run in 70 per cent *n*-propanol at 25°C for about 3½ hrs. Since the filter paper had a size of 16.1 × 22.8 cm, 10 samples could be run on the same sheet. After drying at room temperature the paper was sprayed with 1 per cent KOH in absolute methanol and again dried at 60°C for 20 minutes. The stripes which contained the ninhydrin-positive substances were cut out and placed individually into test tubes. The color reaction was made in a buffer solution according to the modified method of Boissonnas [2], and the color intensity estimated with the Beckman spectrophotometer at a wavelength of 570 mμ. In preliminary tests the urodele embryos were found to con-

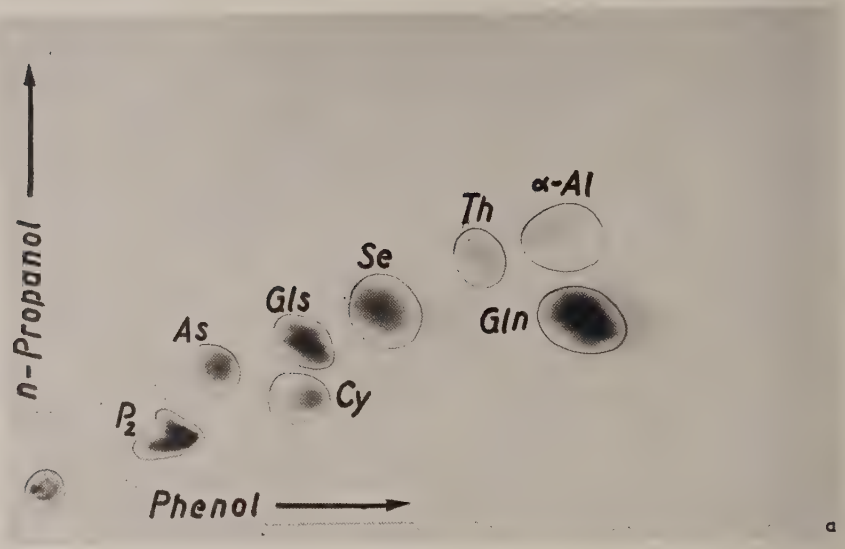
tain high concentrations of free amino acids and peptides so that two eggs were sufficient for a single determination.

For the study of individual amino acids and peptides 20 embryos were prepared by the method referred to above, mashed in a small glass tube and extracted in 80 per cent methanol. The samples were centrifuged and only the supernatant was transferred to the filter paper (28.6 × 46.2 cm) for two-dimensional chromatography. The first solvent used was 70 per cent *n*-propanol and the second one water-saturated phenol. For qualitative analysis the chromatogram was sprayed with 0.1 per cent ninhydrin in absolute ethanol, and the color developed at 90°C for 15 minutes. Procedures used for quantitative determinations were the same as those described above for estimation of the total amount except that the spots were first located by spraying the paper with a 0.01 per cent ninhydrin solution. The correction factors previously worked out by Stumm-Zollinger [30] and by Chen and Hadorn [4] were used in calculating the quantities of different amino acids from their extinction values.

RESULTS

Qualitative analysis.—The two-dimensional chromatograms showed that early blastulae of *Triton alpestris* contain the following free ninhydrin-positive substances: aspartic acid, glutamic acid, serine, threonine, cystine, α-alanine, glutamine and one peptide (Fig. 1*a*). In accordance with the observation reported earlier [5], new amino acids and peptides appear as development proceeds, and these are in a higher concentration at later stages. For instance, in the newly hatched larvae there have been identified, in addition to those already present in the early blastulae, 14 more free amino acids and peptides: glycine, tyrosine, valine, methionine, leucine, tryptophan, γ-aminobutyric acid, proline, histidine, lysine, arginine and three peptides (Fig. 1*b*). Spots N_1 and N_2 in the sketch given by Chen and Rickenbacher [5] have been identified to be peptides, and spots N_3 and N_4 cystine and glutamine respectively. Spot *P*, tentatively designated polypeptide complex in our earlier work, was not recorded here. Repeated tests showed that it appeared on the two-dimensional chromatogram only when both extract and protein precipitates were transferred to the filter paper. As mentioned previously, the supernatant alone was used for chromatography in the present study.

Tryptophan appeared only as a faint spot on the two-dimensional chromatogram. However, it formed a distinctly blue-purple spot after spraying the one-dimensional chromatogram with a 1 per cent *p*-dimethyl-benzaldehyde solution. With the solvents used in the present experiment, valine and methionine cannot be separated satisfactorily; but the platinic iodide test, which is specific for S-containing amino acid, confirmed that this substance is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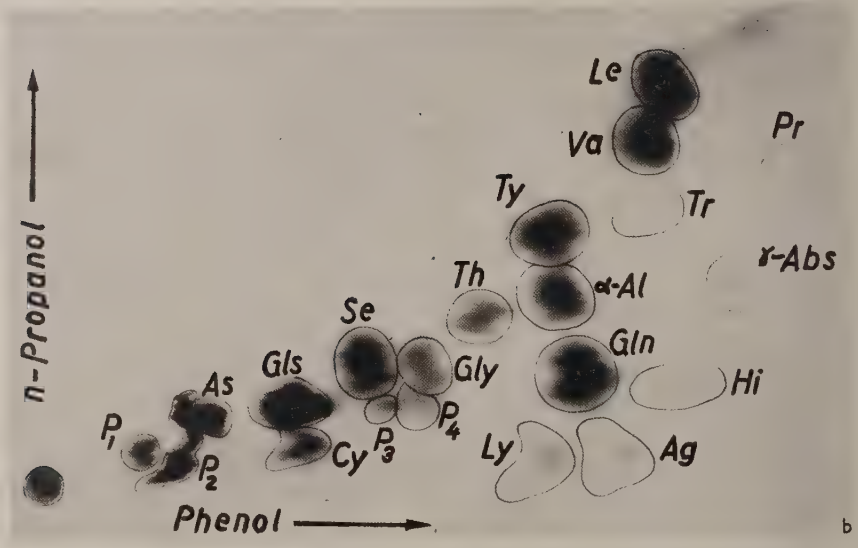
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Gly
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Hi
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P2
Cy
P3
P4
Ly
Ag
b

present in detectable quantity. Spot γ-Abs in Fig. 1*b*, according to its R_f-values and our earlier experiences from experiments on various insects [4], has been tentatively designated γ-aminobutyric acid. No specific test was carried out to check the identification of this amino acid. Glutamine is one of the most abundant free ninhydrin-positive substances, and disappeared from the chromatogram by subjecting the extract to acid hydrolysis in 6.N

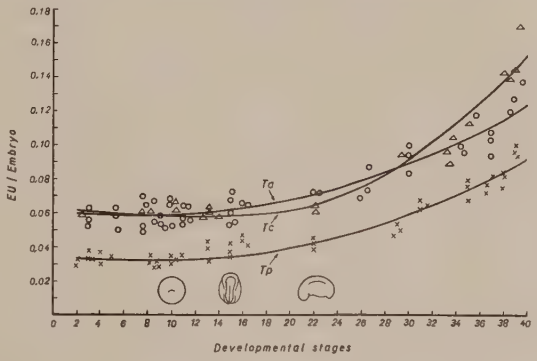

Fig. 2. Changes in total free amino acids, amide and peptides during the urodele development. The curves were drawn according to the method of regression on two variates. Ordinate: extinction unit (EU) per embryo. Abscissa: Harrison stage of development. ○ = *Triton alpestris* (*Ta*); × = *Triton palmatus* (*Tp*); △ = *Triton cristatus* (*Tc*).

HCl at 110°C for 12 hrs. Its disappearance was reflected in the intensification of glutamic acid. Finally, two other amino acids, phenylalanine and ornithine, which were not found in unhydrolyzed extract, formed two distinct spots on chromatograms of the hydrolysate.

Besides the amino acids referred to above, at least four peptides have been identified (spots P_1–P_4 in Fig. 1*b*). Like glutamine, they all disappeared upon hydrolysis. Peptides 1, 2 and 3 have a blue-purple and peptide 4 a brown color on chromatograms sprayed with ninhydrin solution. Peptide 3 occurred in a minute quantity and could only be observed occasionally. Peptide 2, which had by far the highest concentration, was eluted from the chromatogram and hydrolyzed alone. Further analysis showed that the hydrolysate contained at least aspartic acid, glutamic acid, serine and glycine.

Quantitative analysis.—In all three urodeles there is a steady increase of total free ninhydrin-positive substances as development proceeds. The regression curves in Fig. 2 show that the increase begins in early gastrulation

Fig. 1 *a* and *b*. Chromatograms of free ninhydrin-positive substances in alcoholic extracts of 20 *Triton alpestris* embryos. *a*, Early blastulae (Harrison stage 7). *b*, Newly hatched larvae (Harrison stage 40). *As*, aspartic acid; *Gls*, glutamic acid; *Cy*, cystine; *Se*, serine; *Gly*, glycine; *Th*, threonine; *Ty*, tyrosine; *α-Al*, α-alanine; *Gln*, glutamine; *Ly* lysine; *Ag*, arginine; *Hi*, histidine; *γ-Abs*, γ-aminobutyric acid; *Tr*, tryptophan; *Pr*, proline; *Va*, valine (+methionine); *Le*, leucine; P_1–P_4, peptides.

TABLE I

Average fresh weights of *Triton alpestris* during embryonic development.

Development (Harrison stage)	Fresh weight in mg/embryo	
	n	*M* ± S.E.
5–6	17	4.75 ± 0.206
30	9	4.03 ± 0.304
39–40	9	4.71 ± 0.305

TABLE II

Average dry weights of *Triton alpestris* during embryonic development.

Development (Harrison stage)	Dry weight in mg/embryo	
	n	*M* ± S.E.
2–3	17	1.01 ± 0.071
5–7	16	0.99 ± 0.061
9	11	1.14 ± 0.051
13–14	23	1.03 ± 0.031
25–26	12	0.96 ± 0.041
30	14	1.02 ± 0.029
32–33	24	1.04 ± 0.011
39–40	14	1.03 ± 0.012

The dry weight of amphibian embryos consists largely of proteins, which remain constant in the early development as shown by data of total N determinations [14, 25, 32]. According to Gregg [14] there is a loss of glycogen due to combustion in *Rana pipiens* embryos. Løvtrup [25] has also shown that carbohydrate and fat serve as the main energy sources in the embryogenesis of *Amblystoma mexicanum*. In the present study the variation of egg samples used for weight determinations apparently obscured the slight drop of these substances.

and becomes evident during neurulation. In later development there is a more rapid rise in their concentration. Before stage 30 the pattern of change in all three species is the same. However, prior to hatching, there is apparently a greater increase in *Triton cristatus* than in the other two urodeles.

There is no doubt that the steady increase of protein synthesis in differentiating tissues accounts for the rise of the amino acid content. There is evidence that the total quantity of N-containing materials within the embryo does not change significantly, and these do not serve as energy source. According to the work of Gregg and Ballentine [15] on *Rana pipiens* embryos, values of total N remained the same throughout development and showed a slight

increase only after Shumway stage 19. The recent study of Løvtrup [25] on *Amblystoma mexicanum* embryos has confirmed the fact that total N remains essentially constant during early embryonic life. In the present study no estimation of total N was carried out. However, determinations of both fresh and dry weights on *Triton alpestris* embryos also indicated that no detectable changes took place within the observed development period (Tables I and II).

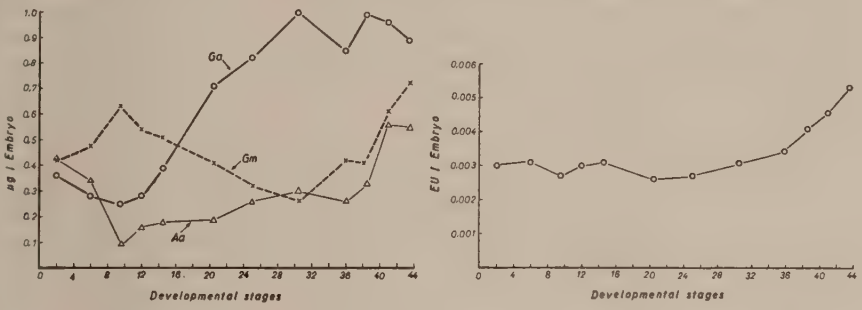

Fig. 3 (*left*). Changes in free aspartic acid (*Aa*), glutamic acid (*Ga*) and glutamine (*Gm*) during the development of *Triton alpestris*. Each point presents the average value of at least two determinations. Ordinate: μg substances (γ) per embryo. Abscissa: Harrison stage of development.
Fig. 4 (*right*). Changes in a free peptide (P_2) during the development of *Triton alpestris*. Ordinate: extinction unit (EU) per embryo. Abscissa: Harrison stage of development.

Quantitative changes of aspartic acid, glutamic acid and glutamine are summarized in Fig. 3. With the beginning of cleavage both aspartic acid and glutamic acid fall rapidly until a minimum is reached at the end of blastulation. Thereafter they show a steady increase until stage 30, decrease slightly at stage 36, and again rise to a maximum about the time of hatching. The general pattern of change is the same for both amino acids, with the exception that before stage 6 the content of aspartic acid is somewhat higher than that of glutamic acid. The opposite is true during later development. Glutamine behaves, however, in the reverse manner. At stage 10, where aspartic acid and glutamic acid drop to a minimum, this amide rises to a level which is even comparable to newly hatched larvae. It shows a minimum at stage 30, whereas a definite increase of the two amino acids was observed at this particular stage. The glutamine curve therefore presents a mirror image of the two curves plotted for aspartic acid and glutamic acid. The significance of this fact will be discussed later.

The extinction values of peptide 2 are presented in Fig. 4. It exhibits again a slight decrease in concentration at the onset of gastrulation. However,

unlike the two amino acids referred to above, no further decline was observed. The observed values indicate a consistent increase in concentration of this substance at all developmental stages ranging from early embryo to the hatched larvae.

Preliminary trials showed that in the early development the content of other amino aicds is low. Detailed determinations of these acids were there-

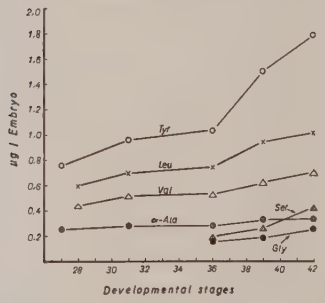

Fig. 5. Changes in free tyrosine (*Tyr*), leucine (*Leu*), valine (*Va*), α-alanine (α-*Ala*), serine (*Ser*) and glycine (*Gly*) during the late embryonic development of *Triton alpestris*. Ordinate: µg substance per embryo. Abscissa: Harrison stage of development.

fore limited to embryos which had reached Harrison development stage 26. Fig. 5 presents the average values of tyrosine, leucine, valine, α-alanine, serine and glycine. The plotted curves show that the behavior of all these amino acids is very similar. There is a slight increase up to stage 36, followed by a more rapid rise shortly before hatching. One point which deserves special mention is the fact that the tyrosine content becomes particularly high when the pigmentation of the embryo begins. It is known that this amino acid serves not only as the precursor of such hormones like adrenaline and thyroxine, but also as the raw material in the formation of melanin.

DISCUSSION

Results of the present study, together with those of earlier investigations, show that there are some differences in free amino acid and peptide content between urodele and anuran embryos. In *Rana pipiens* embryos the following free amino acids have been found: aspartic acid, glycine, histidine, threonine, arginine, tryptophan, valine, glutamic acid, phenylalanine, glutamine, lysine, tyrosine, serine, alanine, methionine sulfoxide (?) and leucine (or isoleucine) [8, 22]. Deuchar [7] reported that embryos of *Xenopus laevis* contain free aspartic acid, glutamic acid, glycine, arginine, valine, α-alanine, β-alanine, leucine, cystine and glutamine. In *Triton alpestris* blastulae only 5 of these 10 amino acids could be observed, namely aspartic acid, glutamic

acid, cystine, α-alanine and glutamine. On the other hand, serine, threonine and a hydrolyzable substance are present in detectable quantity. The results of Deuchar's work are comparable to those of the present study, because in both experiments a similar technique was used for the separation of the amino acids. It seems, therefore, that these differences are of a specific nature. However, considering that the free ninhydrin-positive substances in the same species vary greatly at different development stages and that the amount of material used for the preparation of extract was not the same, it is difficult to make a strict comparison and to ascertain the real significance of such differences.

Another point which has attracted the interest of various investigators is how the changes in free amino acids and peptides are related to the morphogenetic processes. There have been several experiments suggesting the close relation of protein synthesis to tissue differentiation. Friedberg and Eakin [12] demonstrated that there was an increasing uptake of radioactive glycine in *Hyla regilla* embryos from gastrula to larval stages. Furthermore, the incorporation was greater in the dorsal than in the ventral halves. The same was true of the uptake of radioactive methionine in *Rana pipiens* gastrulae [9]. Deuchar [7] also observed that in *Xenopus laevis* embryos the dorsal halves of blastulae, early gastrulae and late neurulae have a higher concentration of total free amino acids than have the ventral halves. Experimental data of total free amino acids and peptides in the present study clearly indicate that the increase of these substances begins at gastrulation, and becomes more rapid in later development when the organogenic events are in full swing. According to Gustafson and Hjelte [16] the synthesis of protein in sea urchin embryos also begins about the time of gastrulation.

Further determinations of free aspartic acid, glutamic acid, glutamine and peptide showed that the behavior of each individual substance varies. Neither a general increase nor an overall pattern of change was observed. Both aspartic acid and glutamic acid drop to a minimum at late blastula stage and rise again during most of the later development. According to Deuchar [7] these two amino acids also decrease between morula and late gastrula stage in *Xenopus laevis* embryos. Glutamine rises to a high level at late blastula stage, declines afterwards until late embryonic life, and rises again. A similar decrease of this amide was recorded in *Xenopus laevis* embryos between late neurula and early tailbud stages. No data were given for still younger embryos.

Aspartic acid, glutamic acid and glutamine maintain a high concentration throughout the embryonic development of *Triton alpestris*. According to

experimental results so far reported, these three substances are of primary importance in protein synthesis. They play an essential part in transdeamination and transamination. Because of the abundance of glutamic enzyme in animal tissues, glutamic acid can be formed through the reaction of α-ketoglutaric acid with the α-amino group of any amino acid. Under appropriate conditions it undergoes deamination and releases ammonia for the synthesis of other amino acids [3]. Glutamine is an amide of glutamic acid and its formation, an endergonic reaction, necessitates the participation of ATP [10, 29]. Series of recent investigations have shown that this important amide is involved in many biological processes. First, it has the function of detoxicating aromatic acids and possibly also ammonia [31]. Second, it serves as a store of the amino group. Its α-amino group can be transferred to other α-keto-acids by the process of transamination [26]. Third, it takes a part in the Krebs cycle, especially in the formation of citrulline [1]. Finally, according to evidence produced by current reserch, glutamine is primarily involved in protein synthesis by the process of transpeptidation [13, 19].

It is worth noticing that in *Triton alpestris* embryos, glutamine shows a high concentration when both aspartic acid and glutamic acid drop to a minimum at late blastulation. Apparently between early morula and late blastula stages glutamine is formed at the expense of these amino acids. The situation is, however, reversed in the later development. In the elongating embryos the concentration of glutamine falls to a minimum while that of glutamic acid becomes very high. The possible explanation is that during this period glutamine is broken down and its amino group is set free for the synthesis of other amino acids. Glutamic acid is, therefore, again regenerated. Unlike glutamine, the peptide exhibits a steady increase except for a slight drop at the beginning of gastrulation. It seems that this peptide does not play a mediating role in the biosynthetic process, but is itself a precursor of the proteins formed in differentiating tissues. Since the quantitative determinations of the present study have been limited only to those substances which are present in high concentration, it is not yet possible to give a full account of the intricate events of protein synthesis in amphibian development. The interpretation of experimental data has to be reexamined when further information is available.

SUMMARY

1. Metabolic changes in free amino acids and peptides during the development of three urodeles, *Triton alpestris*, *Triton palmatus* and *Triton cristatus*, were studied by the method of paper partition chromatography.

2. In the early blastulae of *Triton alpestris* the following free ninhydrin-positive substances have been identified: aspartic acid, glutamic acid, cystine, serine, threonine, α-alanine, glutamine, and one peptide. In the newly hatched larvae the free amino acids and peptides are present in a much higher concentration. In addition to the substances detected in the early blastulae, they contain free glycine, tyrosine, lysine, arginine, histidine, tryptophan, γ-aminobutyric acid, valine, methionine, leucine, proline and at least three more peptides.

3. Quantitative determinations of total free amino acids and peptides performed on embryos of all three urodele species showed that the increase of these substances starts at the onset of gastrulation, becomes more obvious during neurulation and rises rapidly in later embryonic development.

4. Aspartic acid and glutamic acid fall to a minimum at the late blastula stage, increase steadily afterwards and again show a slight drop prior to hatching. Glutamine behaves in the opposite manner. It rises to a high concentration at the late blastula stage, then decreases to a low level and reaches a maximum in the newly hatched larvae. With the exception of a minor drop at the early gastrula stage, the peptide shows a steady increase in concentration as development proceeds. Finally, a general increase of tyrosine, leucine, valine, α-alanine, serine and glycine was detected during later development. The concentration of tyrosine rises more rapidly as the pigmentation of the embryo becomes obvious.

5. The significance of the experimental results in relation to morphogenetic processes and their bearing on problems of protein metabolism are discussed.

The author is greatly indebted to Prof. Dr. E. Hadorn for his interest in the progress of this work and for his valuable criticism in the preparation of the manuscript. Thanks are also due to Dr. C. Zeller for his help in the mathematical treatment of the experimental data.

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STUDIES IN HISTOCHEMISTRY

XXXIX. THE PERFORMANCE OF FREEZING-DRYING APPARATUS FOR THE PREPARATION OF EMBEDDED TISSUE AND AN IMPROVED DESIGN[1]

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In an earlier communication from this laboratory detailed descriptions were given of simple and efficient freezing-drying apparatus for the preparation of embedded tissue [6]. Since that time an impressive number of papers have appeared dealing with further modifications of freezing-drying equipment, and most of these have been considered in recent comprehensive reviews, Neumann [12], Bell [1], Kanehira [10], and Gersh and Stephenson [5]. On the basis of theoretical treatments, Stephenson [13, 14], Gersh and Stephenson [5], and Freed [4] have concluded that the lower pressures obtained with diffusion pumps do not improve performance significantly and that the requirements of the apparatus, in general, are not especially critical. According to Gersh and Stephenson, "In order to dry a few pieces of frozen tissue, vacuum and refrigeration equipment costing thousands of dollars is not necessary. Just as good results can be obtained by drying the tissues in a refrigerated test tube evacuated with a mechanical pump". The experience in our laboratory is in accord with this statement provided that the time of freezing-drying is not a consideration, and by the use of apparatus a little more complex than a test tube, we have succeeded in reducing the dehydration time from days, or in some cases weeks, to a matter of hours. The time seems ripe to report an experimental study of the practical value of certain features in the design of commonly used apparatus in order to demonstrate what simplifications can be justified.

This communication is concerned with an experimental investigation of the effects of certain conditions of temperature and pressure on the time required to freeze-dry standardized small tissue samples in a given apparatus.

[1] Aided by an Institutional Research Grant from the American Cancer Society, The Medical Research Fund of the Graduate School of the University of Minnesota, and research grant No. RG-3911 (C2) from the National Institutes of Health, United States Public Health Service.

In addition, an improved liquid gas trap containing a Dewar reservoir has been designed for freezing-drying equipment and this will be described in detail.

EXPERIMENTAL

Index of Completeness of Dehydration

The best index of the completeness of dehydration is attainment of constant sample weight. For this purpose Jansen [7] devised a torsion balance which could be attached to the freezing-drying apparatus. However, we have observed that without special equipment a practical index of dehydration is given by the length of time bubbles arise from the tissue after it has been submerged in the molten paraffin used for embedding *in vacuo*, plus a microscopic examination after the tissue has been sectioned, deparaffinized, and stained. The justification of this index has been established by tests on hundreds of tissue samples over a six year period, and the results presented in this paper are illustrative.

Apparatus

For comparative studies of performance under different conditions, the unit described in the addendum to the original report [6] was used because it proved to be the most generally useful of the units designed and tested in this laboratory at that time (Fig. 1).

A difficulty which is encountered in the use of this unit is that the highly

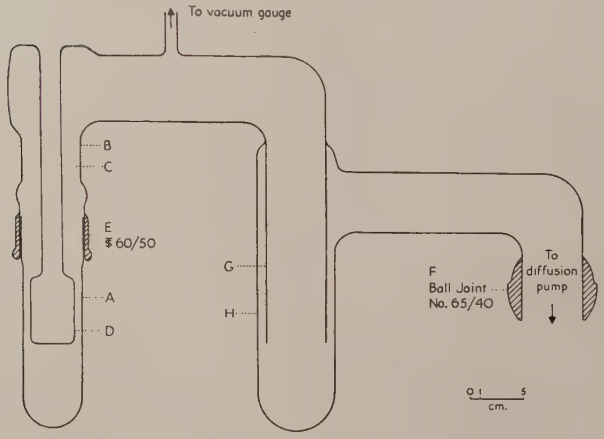

Fig. 1. All-glass freezing-drying unit of Glick and Malmstrom [6].

efficient "cold finger" trap (*D*) placed just above the sample has to be refilled with liquid nitrogen about every hour, and for samples which need overnight treatment it is more convenient to use the other, but less efficient, trap (*H*) which is cooled by liquid nitrogen in a large Dewar flask. To retain the advantages of the "cold finger" trap, while eliminating the disadvantage of frequent refilling, a modification has been introduced in which the "cold finger" consists of a copper tube which is an extension of a copper Dewar reservoir with a 5 liter capacity. The new design has proved to be both more convenient and more efficient than the older one.

The improved model is shown in Fig. 2. This is an experimental unit which was designed to test operation under different conditions, and, as will be shown, certain simplifications can be introduced for a practical laboratory apparatus. The forepump (*P*) is a 2 stage electric motor-driven pump with a free air capacity of 33.4 l/min. (Duo-Seal, No. 1405 H, W. M. Welch Scientific Co., Chicago, Ill.). The oil diffusion pump (*O*) is a water-cooled pump (No. VMF-50W, Consolidated Vacuum Corp., Rochester, N.Y.) with a capacity of 50 l/sec. at 1×10^{-4} mm Hg using "Octoil-S" fluid, and it gives optimal performance when the heater is operated at 110 volts A.C. A "Veeco" vacuum valve (*V*) (Vacuum Electronic Engineering Co., New Hyde Park, Long Island, N.Y.) is sealed to the diffusion pump to permit the vacuum to be broken in the apparatus without turning off the pumps. The needle valve (*N*), attached by plastic tubing to drying tube (*A*) which is filled with anhydrous calcium sulfate ("Drierite"), serves to admit dry air to the system when the vacuum is to be broken. The glass parts (*C*, *B*, *T*) are made of "Double Tough" Pyrex glass (Corning Glass Works, Corning, N.Y.), 3 inch diameter, which is so durable that it can even be dropped on a cement floor without breaking. The Philips vacuum gauge (*G*) (Type PHG-1, Consolidated Vacuum Corp.) is sealed to a brass collar (*I*) connecting (*C*) and (*B*) and the gauge can be removed by unscrewing the knurled circular nut that is placed between it and the collar. The Philips gauge meter box (*M*) contains a meter that registers pressures down to 1×10^{-5} mm Hg.

The Dewar trap (*D*), made to order by Hofman Laboratories Inc., Newark, N.J., has a 5 l. capacity and is constructed of 2 concentric spherical copper shells with the outer one soldered to a brass flange. The internal surfaces of the shells are highly polished and the shells are separated by rope bumpers. The "cold finger" is a downward extension of the inner shell that holds the refrigerant, and both shells have tubular extensions rising above the spherical portions. The top of this upward extension of the inner shell is attached to a spring suspension carried in the outer shell. The design pre-

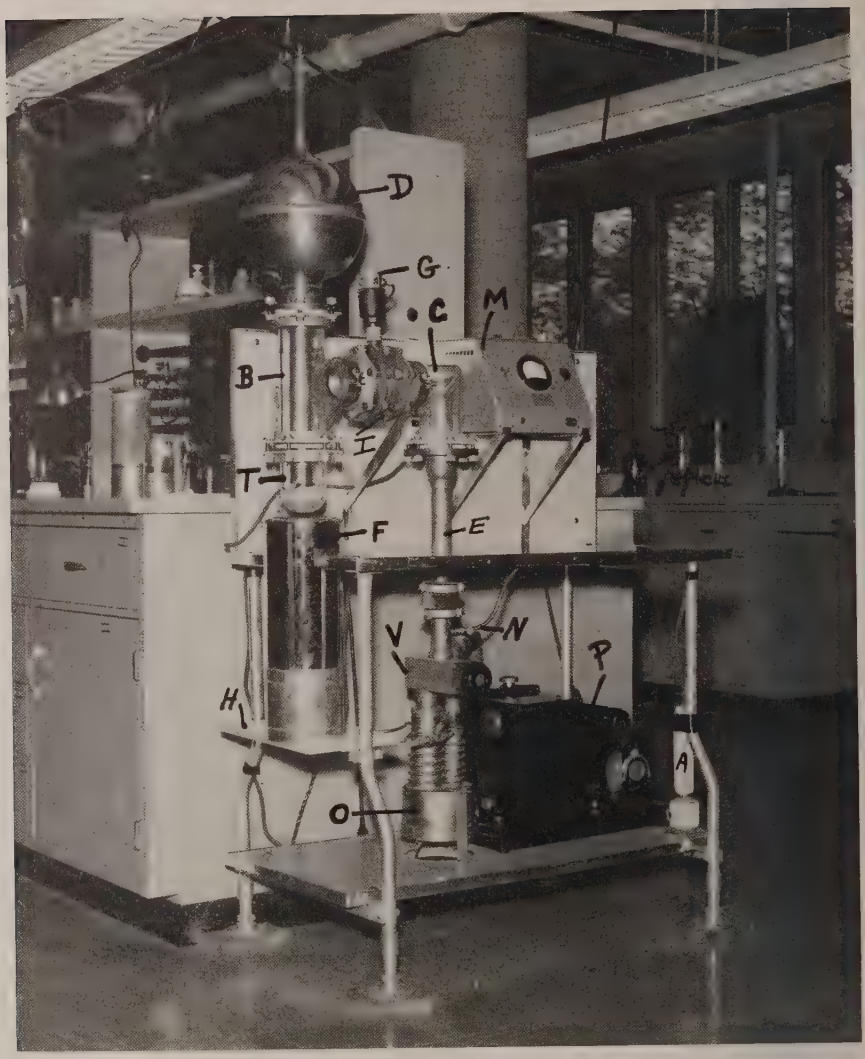

Fig. 2. Complete assembly of freezing-drying unit with Dewar reservoir and copper "cold finger".

sented to the Hofman Laboratories called for the space between the two shells to be completely sealed off and evacuated to a pressure of 1×10^{-5} mm Hg or less, to assure efficient operation of the Dewar reservoir. This feature was omitted, however, and the top of the "cold finger" tube was not sealed to the outer shell so that vacuum between the shells depends on the vacuum produced in the system during operation. (Therefore the liquid N_2 is not poured into the reservoir until the apparatus has been evacuated.) Nevertheless, rather good preservation of the liquid N_2 was maintained during operation even so, one filling of the reservoir lasting about 24 hours with only the forepump running (pressure in system 1×10^{-4} to 5×10^{-4} mm Hg) and, of course, longer when the diffusion pump was used (0.1×10^{-5} to 1×10^{-5} mm Hg).

All glass parts which are to be sealed to metal parts are fitted with aluminum flanges containing asbestos inserts (Corning Glass Works). The metal parts are soldered to corresponding brass flanges. The flanges are bolted together with a teflon gasket between the glass and metal surfaces to secure the seal. Thus (*D*) is connected to (*B*), (*B*) to (*T*), (*B*) to (*I*), (*I*) to (*C*), and (*C*) to the copper pipe (*E*). The diameters of the flanges and the copper pipe are 3 and 1.5 inch, respectively. From Fig. 1 it is apparent that the Dewar flask (*F*) rests on a wooden platform (*H*) to which clamps are screwed at each end. The platform is movable vertically and can be set at a given level by wing nuts that operate the clamps which surround, and are supported by, two of the tubular legs of the table that holds the apparatus.

Comparison with Similar Equipment Recently Described from Other Laboratories

Moberger *et al.* [11] employed a design essentially the same as that illustrated in Fig. 1, but with a metal tube connection between the sample and trap tubes. (These authors devised an electrical induction method for melting the paraffin after dehydration.) Butler and Bell [2] described a "cold finger" type of all-glass apparatus with a relatively large liquid gas capacity, and Freed [4] and Eränkö [3] employed units similar to the earlier model, Unit No. 2 [6], used in this laboratory, but without vacuum gauge or diffusion pump. The traps in the apparatus of both Freed and Eränkö can be used with either a liquid gas or solid carbon dioxide refrigerant, but Freed claimed that on the basis of theoretical considerations no appreciable advantage results from the use of the lower temperatures of liquid gas compared to alcohol-solid carbon dioxide.

The principle of freezing-drying tissue by passing cold dry air over the sample has been proposed by Treffenberg [15] and Jensen [9]. The latter found that this gave a more rapid dehydration than could be obtained with his earlier apparatus [8] which was similar in design to our Unit No. 1 [6]; but a comparison to recent, more efficient units, remains to be made.

Sampling

It is essential that the same number of samples of the same size, shape, and nature be used in experiments which are to be compared. Accordingly, 2 mm cubes of fresh liver, or pieces 2 mm square cut from the same sheet of gastric mucosa about 1 mm thick, were employed. The cutting was made convenient by a device constructed by taping 2 safety razor blades against the flat sides of a plate of wood of such a thickness that the distance between the 2 parallel cutting edges was 2 mm, and in such a manner that the wood offered no obstruction between the blades for a distance of 6 mm up from the cutting edges. By making cuts in 2 or 3 dimensions the squares of mucosa or cubes of liver, respectively, were obtained.

The initial freezing of the tissue in isopentane cooled by liquid nitrogen, the loading and degassing of the paraffin in the sample tube (T), and the procedure followed were virtually the same as described earlier [6].

Operational Characteristics

Distortions of the cellular structures were always obtained when any bubbling occurred, and the longer the bubbling proceeded the greater the distortion found. The preservation of structural characteristics was excellent when no bubbles formed, with the exception that intracellular changes were seen, particularly in the cytoplasm, in the absence of bubbling if the initial freezing of the tissue was faulty or if a low enough temperature was not used during the dehydration.

Data given in Table I reveal the following main points: Comparison of Exp. 1–3 shows that 8 or 9 hours was not a sufficient dehydration time for satisfactory results under the conditions used, but that 10 hours was adequate. However, 10 hours of dehydration was still insufficient when the sample was held at $-78°$ instead of -45 to $-50°$C, regardless of whether the pressure was that of the mechanical pump vacuum (Exp. 5) or of the diffusion pump vacuum (Exp. 6). When the time of the latter experiment was extended to an overnight period, 16 hours, excellent results were obtained (Exp. 7).

TABLE I

Freezing-drying with glass-trap apparatus (Fig. 1).
In each experiment the sample was 2 cubes, each 2 mm^3, of liver from normal male albino mice. Pressures of 35×10^{-5} mm Hg and higher were obtained with mechanical pump alone, lower pressures with oil diffusion pump.
(DO CO_2) Diethyl Oxalate–Solid CO_2, $-45°$ to $-50°$; (A–CO_2) Acetone–Solid CO_2, $-78°$; (N_2) Liquid N_2, $-195°$.

Exp. No.	Refrigerant: Sample tube	Refrigerant: Trap	Hours	Lowest pressure mm Hg. ($\times 10^{-5}$)	Minutes of bubbling	Microscopic appearance after H and E staining
1	DO–CO_2	N_2	8	0.1	20	Extensively vacuolated
2	DO–CO_2	N_2	9	0.5	11	,, ,,
3	DO–CO_2	N_2	10	0.5	0	No vacuoles, excellent morphology
4	DO–CO_2	N_2	10	42.0	1	Only center vacuolated, outer part good
5	A–CO_2	N_2	10	35.0	15	Extensively vacuolated
6	A–CO_2	N_2	10	0.1	15	,, ,,
7	A–CO_2	N_2	16	4.0	0	No vacuoles, excellent morphology
8	DO–CO_2	A–CO_2	10	60.0	11	Extensively vacuolated
9	DO–CO_2	A–CO_2	10	2.5	1	Only center vacuolated, outer part good
10	DO–CO_2	A–CO_2	16	55.0	0	No vacuoles, excellent morphology
11	DO–CO_2	A–CO_2	16	2.0	0	,, ,, ,, ,,

TABLE II

Freezing-drying with copper Dewar trap apparatus (Fig. 2).
In each experiment the sample was 3 cubes of liver, each 2 mm^3, and 3 pieces of gastric mucosa, each 1×2 mm^2 from normal male albino rats. Pressures of 10×10^{-5} mm Hg and higher were obtained with mechanical pump alone, lower pressures with oil diffusion pump. Liquid N_2, $-195°$, used in Dewar reservoir.

Exp. No.	Sample tube refrigerant	Hours	Lowest pressure mm Hg. ($\times 10^{-5}$)	Minutes of bubbling	Microscopic appearance after Hand E staining
12	None	4	0.5	0	No vacuoles, but distorted cytoplasm with extensive granulation, nuclei good
13	,,	9	0.3	0	Same
14	,,	12	0.1	0	Same
15	Powdered CO_2	3	50.0	10	Center vacuolated, outer part good in all pieces
16	,,	6	40.0	0	No vacuoles, excellent morphology throughout
17	,,	14	0.1	0	Same

Furthermore, replacement of liquid nitrogen in the trap by acetone-solid carbon dioxide at either fore-pump or diffusion pump vacuum gave unsatisfactory results, although the latter were not as bad as the former (Exp. 8, 9). When Exp. 8 and 9 were repeated, but for 16 hours, excellent results were found (Exp. 10, 11). When Exp. 3 was repeated, but with fore-pump vacuum, the results were not as good as with the diffusion pump (Exp. 4).

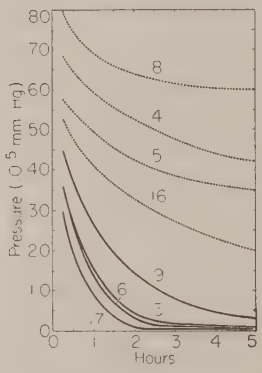

Fig. 3 (*left*). Rates of fall in pressure under different conditions in apparatus illustrated in Figs. 1 and 2. The numbers on the curves refer to the numbers of the experiments in Tables I and II from which the data were taken. The curves drawn with broken lines represent experiments in which mechanical vacuum pumps alone were used, the solid-line curves represent experiments in which oil diffusion pumps were used.

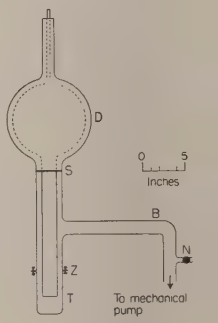

Fig. 4 (*right*). Simplified Dewar reservoir apparatus.

From these data it is obvious that, regardless of the theory one may prefer, for the 10 hour period the colder trap gave better results (compare Exp. 3 with 9, and 4 with 8), and the lower pressures were more advantageous than the higher ones, Exp. 8 and 9, 3 and 4. But it should be quickly added that increasing the time can make the use of a liquid nitrogen trap or a diffusion pump unnecessary (Exp. 10). Of course, when sample was held at a lower temperature the time had to be prolonged to give adequate results, Exp. 7.

Data in Table II show that complete dehydration of samples not subjected to external cooling does not yield satisfactory results, Exp. 12–14. When the samples were cooled with solid carbon dioxide 3 hours with fore-pump vacuum (Exp. 15) was not adequate, but 6 hours gave excellent results (Exp. 16). Obviously, the diffusion pump offered little advantage. The greater efficiency of this apparatus compared with the former can be seen by comparing Exp. 16 with Exp. 5 and 6; the latter gave poor results in 10 hours.

In Fig. 3, the rates of fall in pressure under different conditions in both of the freezing-drying units, Figs. 1, 2, are shown. The data are in accord with the results obtained in the actual tissue dehydration experiments. The slower rates of pressure fall with higher ultimate pressures, obtained by the use of

the mechanical pumps alone, and the faster evacuation rates resulting from colder traps are apparent. Also, the somewhat greater rate of fall in pressure in the improved apparatus can be seen; and this is due primarily to the more efficient Dewar trap, which not only acts as a trap for water but also condenses the oxygen of the air of its surface.

Simplified Apparatus

Although the rate of freezing-drying was found to be greater when a diffusion pump was employed to give a higher vacuum than that furnished by a mechanical pump alone, the saving in time is not sufficient to justify the use of a diffusion pump for most purposes, particularly if an efficient liquid nitrogen trap is employed (Exp. 16). Even the liquid nitrogen trap can be replaced by an acetone-solid carbon dioxide trap if the time of dehydration is prolonged (Exp. 10), and an overnight period is not likely to be too long in most laboratories. Accordingly, satisfactory results with simple designs such as those used by Glick and Malmstrom [6], Butler and Bell [2], Eränkö [3], Freed [4], Jensen [8], and others require only that a long enough time be allowed for the dehydration if a mechanical pump alone and a solid carbon dioxide trap are used.

A compromise in design which retains the advantages of the improved apparatus described earlier in this paper, with simplifications suggested by the tests which have been run, is illustrated in Fig. 4. Referring to Fig. 2, the only part that must be made of glass to permit visibility is the sample tube (*T*). The vacuum gauge (*G–M*) is helpful but it is not essential, the oil diffusion pump (*O*) and the vacuum valve (*V*) are not required. The parts (*B*) (*I*) (*C*) and (*E*) can be replaced by a single metal tube leading directly to the forepump (*P*). The single tube should be supplied with a needle valve (*N*), and, if a vacuum gauge is to be used, an appropriate opening to receive it (*G*). The diameter of the tube should be at least as large as the opening on the mechanical pump up to the vertical part of (*B*), the latter should have a greater diameter to allow an adequate passage between the "cold-finger" and the outer jacket.

SUMMARY

The effect of various experimental conditions on the time required to freeze-dry standardized small tissue samples has been studied. Although more rapid freezing-drying occurred when higher vacua provided by diffusion pumps were used, and colder vapor traps using liquid nitrogen were em-

ployed, the saving in time was often only a matter of hours. Therefore, as reported from other laboratories, the use of a mechanical vacuum pump alone, and a vapor trap filled with a mixture of solid carbon dioxide and acetone or alcohol (−78° C) (in conjunction with a sample tube refrigerant at −30° to −50° C, such as provided with mixtures of solid carbon dioxide with dimethoxytetraglycol or diethyl oxalate, respectively) can be adequate provided that a long enough time is allowed for the freezing-drying process.

For those instances in which the time saving is still a factor, a description has been given of a newly designed liquid gas trap containing a copper Dewar reservoir which has been incorporated in a highly efficient and still relatively simple freezing-drying apparatus.

The authors are indebted to Miss Donna DenBoer for technical help in the present work.

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THE DISTRIBUTION OF PROTEIN-BOUND AZO DYE IN SUBFRACTIONS OF LIVER CYTOPLASM FRACTIONS

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As is well known from the investigations of the Miller group [8, 9, 14], carcinogenic azo dyes, added to the diet of rats, become bound, to a considerable extent, to the proteins of rat liver.

In a previous paper [7] experiments were described in which a single standard dose of the carcinogenic azo dye 3-methyl-4′-dimethylaminoazobenzene (m-DAB) was administered to albino rats. At different intervals after the administration of the dye, the rats were killed. The livers were homogenized and fractionated by differential centrifugation, and the content of protein-bound dye was determined in the cytoplasmic fractions obtained. It was observed in these experiments that during the early period of rapid dye uptake the content of protein-bound dye increased more rapidly in the "microsome" fractions than in any other fraction of the cytoplasm. In this respect, the incorporation of azo dye into the liver proteins resembled the incorporation of labeled amino acids, which also proceeds at a particularly rapid rate in those structures of the cytoplasm which, after homogenization of the tissue, are concentrated in the microsome fraction [3].

With respect to amino acid incorporation the cytoplasmic particles of the liver are not homogeneous. By a combination of extraction and precipitation procedures it was possible to split up such particles into subfractions, containing proteins with different incorporation properties [5, 6]. It was the aim of the present investigation to study the m-DAB incorporation by means of the same subfractionation technique, with the prospect of determining if the amino acid incorporation and the incorporation of carcinogenic azo dye also proceed according to correlated patterns in the sub-particular range.

EXPERIMENTAL

Albino rats weighing 175–200 g were used. Before the experiments they were maintained for 6–8 days on a low protein basal diet [7].

The m-DAB was administered to the rats as a 2 per cent solution in olive oil.

Of this solution 2 ml were introduced into the stomach, and 1 ml into the abdominal cavity of each rat. As a rule, each experiment included 12 rats. For practical reasons, the animals were divided into three groups, which were treated independently. The fractionated material obtained from these groups of animals was subsequently combined for the analyses of protein-bound dye.

In the experiments the following periods of survival after the azo dye administrations were studied: 5, 7, 20, 40 hours, and 5 days. In one experimental series the distribution of bound dye was studied in animals which had been maintained for 3 weeks on a basal diet containing 0.06 per cent of m-DAB.

The techniques used for the homogenization of the livers, and for the differential centrifugation of the liver homogenates have been described in detail elsewhere [7]. Ice-cold 0.25*M* sucrose, containing 0.005*M* versene, was used as the fractionation medium.

The subfractionation of the cytoplasmic fractions was carried out essentially in the same way as in the experiments on amino acid incorporation described previously [6]. The particulate cytoplasmic fractions were treated at pH 8.4 with ice-cold 0.2*M* bicarbonate buffer, and then with 0.4 per cent deoxycholate. The deoxycholate extracts were subsequently fractionated with ethanol at low temperature. As it was felt that slightly increased ethanol concentrations were favourable in the fractionation of proteins from livers with high azo dye contents, the following ethanol concentrations were used in these experiments: (i) 20–26 per cent, (ii) 28–32 per cent, and (iii) 70 per cent ethanol.

Dilute $CaCl_2$ was added to the soluble cytoplasmic fractions until maximum precipitation was obtained [6]. The Ca-precipitates were centrifuged off, and by treatment with versene, a major part of the precipitates were brought into solution. Microsome-like pellets were obtained from these slightly opalescent solutions by centrifugation for 60 minutes at 70,000 g. The supernatant fluids obtained were fractionated with ammonium sulfate (30 and 70 per cent saturation). The cell fluid proteins non precipitated by $CaCl_2$, also, were fractionated with ammonium sulfate (30, 50, 70, and 90 per cent saturation).

As was described in connection with earlier experiments [7], the proteins from the different subfractions were purified by means of a slightly modified Schneider [13] procedure. The contents of bound azo dye in the samples of purified proteins were finally determined according to the method of Miller and Miller [8]. In the majority of fractions enough material was available for allowing the analyses (protein purifications and azo dye determinations) to be carried out in duplicate (50 mg samples).

RESULTS

The main results of the experiments are illustrated by Figs. 1–3. In the experiment represented by Fig. 1 the rats were killed 5 hours after the administration of the m-DAB. As is shown by the figure, the two microsome fractions were in this particular experiment analyzed together. In the experiments illustrated by Figs. 2 and 3 the animals were sacrificed respectively 20 hours and 5 days after the administration of the dye.

In agreement with previous experiments [7] a particularly high content of protein-bound azo dye was, during the early stages of azo dye incorporation, observed in the microsome fractions (Fig. 1, Bar 14). Later, when the deposits of m-DAB had been eliminated, and the content of azo dye in the liver gradually diminished, the cell fluid proteins, non-precipitable with $CaCl_2$, showed the highest content of bound dye (Fig. 3, Bar 26).

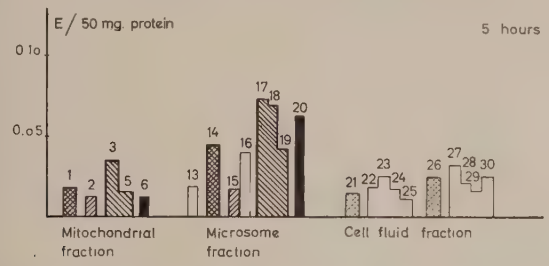

Fig. 1. Contents of protein-bound azo dye in subfractions of cytoplasmic fractions from rat liver. The rats were sacrificed 5 hours after the administration of a single, standard dose of 3-methyl-4′-dimethylaminoazobenzene.

In Figs. 1–3 the subfractions are symbolized in the following way:

Subfractions of cytoplasmic particles:

First bicarbonate extracts: Bars 2, 8, 15.

Second bicarbonate extracts: Bars 16.

First deoxycholate extracts: 20–26 per cent ethanol fractions: Bars 3, 9, 17; 28–32 per cent ethanol fractions: Bars 4, 10, 18; and 70 per cent ethanol fractions: Bars 5, 11, 19.

Non-soluble residues from particles: Bars 6, 12, 20.

Complete particulate fractions: Bars 1, 7, 14.

Microsome washings: Bars 13.

Ca-precipitated cell fluid proteins: Bars 21.

Versene-treated Ca-precipitates sedimenting at 5000 g: Bars 22.

Microsome-like material, sedimenting at 70,000 g: Bars 23.

Ca-precipitated proteins, entirely dissolved in versene: 30 per cent ammonium sulfate fractions: Bars 24; and 70 per cent ammonium sulfate fractions: Bars 25.

Cell-fluid proteins non-precipitable with $CaCl_2$: Bars 26.

30 per cent ammonium sulfate fractions: Bars 27; 50 per cent ammonium sulfate fractions: Bars 28; 70 per cent ammonium sulfate fractions: Bars 29; and 90 per cent ammonium sulfate fractions: Bars 30.

The protein samples which were obtained from the cytoplasmic fractions by means of the subfractionation procedures differed more or less markedly with respect to azo dye content (Figs. 1, 2). Those proteins which were extracted from the cytoplasmic particles by treatment with bicarbonate buffer at pH 8.4 (Bars 2, 8, 15), constantly showed lower contents of bound azo dye than the proteins of the deoxycholate extracts (Bars 3–5, 9–11, 17–19). Within the deoxycholate extracts, the proteins which were easily precipitated with ethanol (Bars 3, 9, 17) had somewhat higher levels of azo dye content than had the proteins which precipitated less readily (Bars 5, 11, 19).

As was shown previously [7], the proteins from the cell fluid fraction, which were precipitated with $CaCl_2$, were characterized by a rather low average content of bound azo dye (Bar 21). It was found, however, that this fraction was very inhomogeneous in respect to azo dye incorporation. A relatively high content of bound dye characterized those proteins of the Ca-precipitates which could not be brought back into true molecular solution

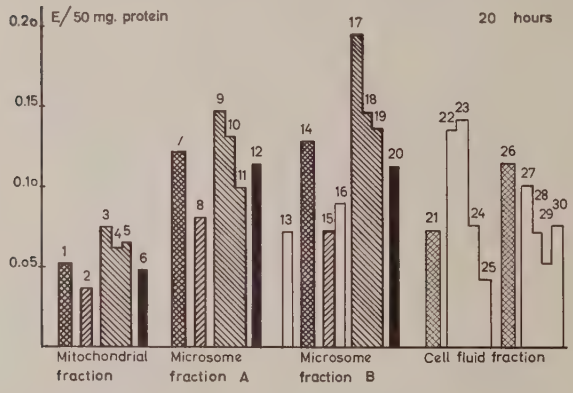

Fig. 2. Experiment of the same kind as in Fig. 1. The rats were killed 20 hours after the administration of the azo dye.

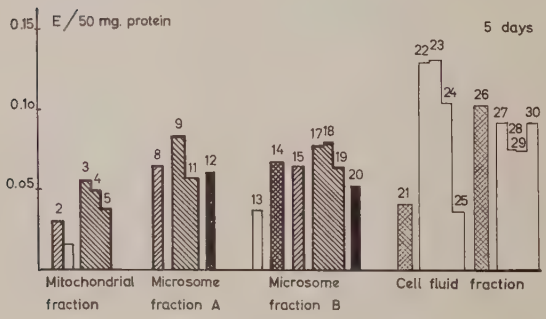

Fig. 3. Experiment of the same kind as in Figs. 1 and 2. The rats were sacrificed 5 days after the administration of the azo dye.

by treatment with versene (Bars 22, 23). This higher content of bound dye was particularly obvious in the proteins of the microsome-like material obtained by centrifugation at 70,000 g (Bars 23). As a contrast, those soluble proteins of the versene extracts which were not precipitated in 30 per cent saturated ammonium sulfate had very low contents of bound dye (Bars 25).

The average content of bound azo dye was considerably higher in the Ca-soluble proteins of the cell fluid (Bars 26) than in the proteins of the Ca-

precipitates (Bars 21). When these Ca-soluble proteins were fractionated with ammonium sulfate, rather high dye contents were observed in the protein fraction, which precipitated at 30 per cent saturation (Bars 27). The proteins which precipitated at 70–90 per cent saturation (Bars 30) were also characterized by fairly high dye contents. It is, however, shown by the figures that the proteins of the ammonium sulfate fractions (Bars 27–30) did not satisfactorily account for the rather high average dye concentration observed in the proteins of the total, Ca-soluble cell-fluid fraction (Bars 26). Possibly some proteins with particularly high contents of bound dye remained in solution in the 90 per cent saturated ammonium sulfate, or behaved in a different way, after the fractionation procedure, at the subsequent treatment with trichloracetic acid and ethanol–ether.

In the experiments of prolonged duration (40 hours, 5 days) the differences in azo dye content between the subfractions gradually became less striking (Fig. 3). As might be anticipated, this was particularly the case in the experiment in which the animals were continually maintained for 3 weeks on a diet containing 0.06 per cent m-DAB.

DISCUSSION

As mentioned above, previous experiments [6] showed that liver cell particles are not homogeneous, but are composed of subordinate proteins with different properties with respect to amino acid incorporation. This inhomogeneity was also clearly demonstrated under the present experimental conditions.

As is shown by Figs. 1 and 2, the primary incorporation of the azo dye into proteins was distributed between the subfractions of the particles in a rather characteristic way. The proteins of the bicarbonate extracts (Bars 2, 8, 15) were brought into solution together with a major part of the ribonucleic acid (RNA) of the particles [6]. These proteins contained relatively small amounts of bound dye. Within the deoxycholate extracts the material which required the highest ethanol concentration in order to precipitate (Bars 5, 11, 19) contained somewhat smaller relative amounts of protein-bound azo dye than did the subfraction which precipitated most readily in ethanol (Bars 3, 9, 17), a subfraction which was fairly poor in RNA.

The incorporation of azo dye was thus distributed between the subfractions of the particles inversely to the amino acid incorporation [6]. While the amino acid incorporation was positively correlated to the RNA-contents of the subfractions, a negative correlation was observed in the case of azo dye

incorporation. The similarity in distribution between the incorporation of amino acids and azo dyes into rat liver proteins, which was observed in the case of whole particulate and non-particulate cytoplasmic fractions [7] did not extend to the subunits of the particles. Obviously, the azo dye incorporation is less intimately tied up with the functions of the nucleoprotein subunits than is the amino acid incorporation. The experiments suggest that the incorporation of azo dye into proteins is mediated by an enzymatic mechanism independent of the amino acid incorporation mechanism, in spite of the fact that both processes are concentrated in particles which accumulate in the microsome fractions.

It has been shown that a number of detoxicating reactions of redox type, including the degradation of dimethylaminoazobenzene, can be carried out by rat liver microsomes under proper conditions [10, 11, 2]. The present experiments are consistent with the notion that the binding of carcinogenic azo dyes to protein is due to irreversible interactions between these very extraordinary substrates and the enzymatic systems responsible for their metabolic detoxication, or to spontaneous reactions between labile intermediate oxidation products and intervening protein molecules [cf. 1]. The possibility of such spontaneous coupling reactions has been demonstrated *in vitro* by Mueller and Miller [12], using glutathione as an acceptor compound. In either case, the microsomal structures, with their vital anabolic functions [cf. 4], are particularly exposed to profound disturbances by the carcinogenic azo compounds.

SUMMARY

Single, standard doses of the carcinogenic azo dye 3-methyl-4′-dimethylaminoazobenzene were administered to albino rats by injection. At different periods after the injections, the livers were homogenized and fractionated by differential centrifugation. The cytoplasmic fractions obtained were separated into subfractions by a technique used previously in analogous experiments with labelled amino acids. Finally, the contents of protein-bound azo dye were determined in the subfractions obtained.

While the incorporation of the azo dye into proteins was distributed between the whole particulate and non-particulate cytoplasmic fractions in approximately the same way as was the incorporation of labeled amino acids, the two incorporation processes were divergent in the subfractions of the particles. While the amino acid incorporation was positively correlated to the RNA-contents of the subfractions, a negative correlation was observed in the case of azo dye incorporation. The results suggest that the azo dye incorpo-

ration is mediated by an enzymatic mechanism independent of the amino acid incorporation mechanism. The experiments are consistent with the notion that the detoxicating enzymatic system in liver microsomes may be involved in the formation of the azo dye-protein complexes.

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ORGANIZATION OF MITOCHONDRIAL DPN-LINKED SYSTEMS[1]

I. REVERSIBLE UNCOUPLING OF OXIDATIVE PHOSPHORYLATION

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The high lability of the mitochondrial enzyme system has constituted a great difficulty in studies of oxidative phosphorylation. Efforts to overcome this difficulty have brought about a great deal of experience about conditions and agents that favor or disfavor a tight coupling between phosphorylation and electron transport, and about characteristic symptoms that are diagnostic of a labilized mitochondrial system. Altered morphological and osmotic properties [17, 18, 7, 8, 39, 40, 34, 6, 38, 1, 19, 27], loss of intramitochondrial nucleotides [37, 4, 43] and appearance of hydrolytic activities [28, 30, 37] are examples of these symptoms.

This body of empirical knowledge has served as a satisfactory basis for achieving a significant level of clarity in some fundamental questions concerning oxidative phosphorylation, such as the number and location of phosphorylations along with the respiratory chain [22, 9, 5, 31]. In all these studies emphasis was laid upon working with stable systems and little attention was paid to the ultimate implications of the concept of mitochondrial stability.

In recent years it has been visualized that the mitochondrial enzyme organization might constitute an operational level of metabolic control mechanisms, e.g. hormones [29, 35, 32, 20, 36]. A reversible labilization of the mitochondrial organization thus appears as a possible physiological reality, focusing interest on a closer examination of the concept of stability, and on a transfer of the empirical experimental facts to a more systematic basis.

Some time ago we commenced an investigation of some of these problems by studying the effects of Ca^{++}, a known labilizer of coupled phosphoryla-

[1] *Abbreviations:* AMP, adenosine-5-phosphate; ATP, adenosine triphosphate; DPN, diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; CoA, coenzyme A; P/O = the ratio of micromoles esterified phosphate to microatoms consumed oxygen.

tion with all the typical concomitant symptoms [44, 37, 40], on the enzymic organization of rat liver mitochondria. In these studies [33] it was found that 0.5–1.0 m*M* Mn^{++} could protect DPN-linked oxidations and oxidative phosphorylations in a Ca^{++}-treated system. This finding could be confirmed subsequently in a number of laboratories, and extended to include protection against enzymic and structural labilization of mitochondria induced by hypotonicity, a series of ions and detergents, and by thyroxine [21, 23, 26, 36, 43, 16].

Another striking effect of Mn^{++} was that, when added to a system previously treated with Ca^{++}, oxidative phosphorylation could easily be restored, provided that the addition was accompanied by a small amount of ATP [33]. This fact, together with the earlier known protective action of ATP on mitochondrial integrity [44, 39, 40], suggested that the Ca^{++}-induced labilization might be due to an exhaustion of mitochondrially bound ATP. An elaboration of these findings in greater detail [13], with the phosphorylations coupled to the aerobic oxidation of succinate as a test system, led to the conclusion that the stability of the coupling between phosphorylation and electron transport in a given mitochondrial system is a function of the level of bound ATP; Ca^{++} promotes the release of ATP from the mitochondrial structure, but this action is antagonized by Mn^{++} or (to a lesser extent) by Mg^{++}, the actual concentration ratios of these ions determining to what degree the occurrence of coupled phosphorylation in a mitochondrial system is dependent upon the ATP concentration in the medium.

Subsequently it could be shown [3] that the variations thus observed in the stability of oxidative phosphorylation were accompanied by reversible changes in the physical state of the mitochondria, as revealed by light scattering measurements. This indicated that the postulated mechanism might have a more general bearing on mitochondrial enzyme organization.

The present work constitutes an extension of these studies to the role played by ATP in the mitochondrial organization of the DPN-linked oxidative system. In Part I evidence is presented that the loss of the mitochondrial complement of DPN, occurring upon Ca^{++} treatment, is a secondary consequence of a release of mitochondrial ATP, and it is shown that this effect ensues more readily than the loss of the phosphorylative ability; the reconstruction of the latter by ATP and Mn^{++} is investigated in some detail. In Part II, the problem of restoring the mitochondrial complement of DPN by treatment with ATP and Mn^{++} will be examined, with special reference to the role of this mechanism in regulating alternative electron transfer pathways.

EXPERIMENTAL

The experimental procedure was principally the same as described previously [13].

Twice washed rat liver mitochondria were prepared in 0.25*M* sucrose [41]; with the exception of one experiment (cf. Table VI), no versene was used. Mitochondria from one liver (containing about 30–40 mg protein-N) were suspended in 20 ml of a solution (pH 7.5) containing 174 mg K_2HPO_4, 230 mg KCl, 216 mg glucose, and AMP or ATP, in concentrations specified in the individual experiments. $MgCl_2$, $MnCl_2$, $CaCl_2$ and hexokinase were added, where indicated, and the suspension was diluted with 0.25*M* sucrose to a final volume of 30 ml. This will be referred to in the following as *standard suspension*, the concentrations of the bivalent cations and of hexokinase being specified in each case.

The term *preincubation* will be used to mean an incubation of a standard suspension in the absence of oxidizable substrate. Preincubations were performed in Warburg vessels on 1.5 ml aliquots of the standard suspension, when the effect of varying cation or hexokinase concentrations was to be tested; when the time of preincubation was varied, the preincubations were performed in 200 ml Erlenmeyer flasks and 1.5 ml aliquots were removed at suitable intervals and transferred into Warburg vessels for test incubation. Substrate and desired individual additions, adjusted to a final volume of 0.5 ml, were added from the side arms of the Warburg vessels at the onset of the test incubation. Preincubations and test incubations were carried out at 30°C under shaking in air.

Oxygen and phosphate uptakes were determined as described previously [13].

Yeast hexokinase was prepared according to Berger *et al.* [2], with minor modifications [13]. The amount of hexokinase used in the individual experiments will be given in reference to the titration curve in Fig. 1. ATP (crystalline sodium salt, Sigma), AMP (crystalline, Sigma), DPN (95 per cent, Boehringer), and cytochrome *c* (Sigma) were commercial products.

RESULTS AND DISCUSSION

The intact system.—In Fig. 1 the tightly coupled state of the intact system is demonstrated, as tested under conditions favoring maximum efficiency [9]. Thus, in the presence of orthophosphate, ATP and 4 m*M* Mg^{++}, and with L-glutamate as substrate, respiration is greatly dependent on the presence of an adequately active terminal phosphate acceptor, in the present case hexokinase-glucose. Alternatively, respiration can be brought to maximum capacity by uncoupling phosphorylation with 2,4-dinitrophenol (Table I).

Effect of Ca^{++} on respiration.—As shown in Table I, 0.25 m*M* Ca^{++} causes a marked decrease of respiration. This effect does not appear in the presence of DPN and is essentially prevented by 0.5 m*M* Mn^{++}. The data suggest that Ca^{++} acts by inducing a release of the mitochondrial complement of DPN and that this action is antagonized by Mn^{++}. The phosphorylative

efficiency, as expressed in terms of P/O ratio, is but marginally affected in the Ca^{++}-inhibited system; this, together with the fact that Mn^{++} exhibits its protecting action on respiration even if phosphorylation is suppressed by dinitrophenol, indicates that the Ca^{++}-induced release of DPN is not a secondary consequence of a decreased phosphorylative capacity.

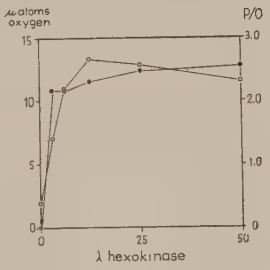

Fig. 1. Effect of hexokinase on respiration in intact mitochondria. *Circles*, oxygen uptake; *points*, P/O ratio. Standard suspension of mitochondria (cf. Experimental), containing ATP (1.5 m*M*) and Mg^{++} (4 m*M*). Substrate, L-glutamate (15 m*M*). Microliters of hexokinase per vessel, as indicated. Time of incubation, 15 min.

TABLE I

Effect of Ca^{++} on respiration under varying conditions.

Standard suspension of mitochondria, containing ATP (1.5 m*M*) and Mg^{++} (4 m*M*). Additions (where indicated): Ca^{++} (0.25 m*M*), Mn^{++} (0.5 m*M*), hexokinase (25 microliters per vessel), 2,4-dinitrophenol (0.2 m*M*), DPN (0.8 m*M*). Substrate, L-glutamate (15 m*M*). Time of incubation, 15 min.

Additions	Oxygen uptake microatoms	P/O
None	2.9	
Hexokinase	12.6	2.88
Hexokinase, Ca^{++}	4.5	2.11
Hexokinase, Ca^{++}, DPN	12.1	2.67
Hexokinase, Ca^{++}, Mn^{++}	10.5	2.43
Dinitrophenol	13.8	0.07
Dinitrophenol, Ca^{++}	6.7	0.05
Dinitrophenol, Ca^{++}, DPN	13.5	0.08
Dinitrophenol, Ca^{++}, Mn^{++}	12.8	0.08

It has been found previously [33] that, under more drastic conditions than those applied above, Ca^{++} treatment also may result in an inactivation of phosphorylation and that, even in such a case, Mn^{++} can exhibit full protection. It appeared improbable, on the other hand, that, conversely to what could just be ruled out, the loss of the phosphorylative ability would

be a secondary consequence of a loss of the mitochondrial DPN, since the former did occur even in the succinic oxidase system where no DPN is involved [12, 13]. The protective action of Mn^{++} against any of the two effects when present separately suggested a common mode of action of Ca^{++}. As such an action the release of mitochondrial ATP has been postulated [13].

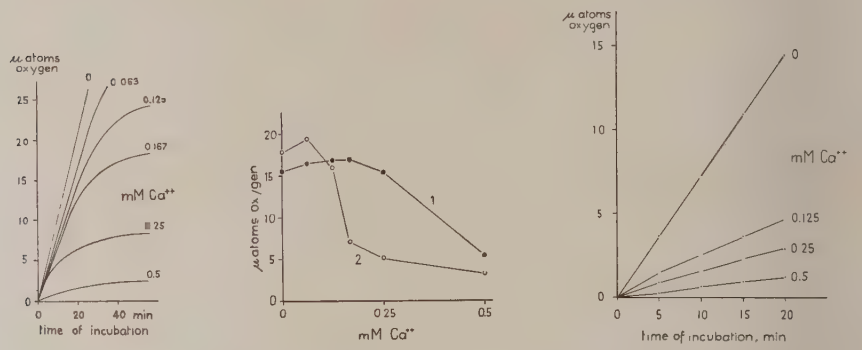

Fig. 2 (*left*). Effect of varying concentrations of Ca^{++} on respiration. Conditions as in Fig. 1, except that the amount of samples per vessel was increased 1.5 times. Each vessel contained 37.5 microliters hexokinase.

Fig. 3 (*centre*). Effect of terminal phosphate acceptor on Ca^{++} sensitivity of respiration. Standard suspension of mitochondria, containing AMP (10 m*M*) and Mg^{++} (4 m*M*). *Curve 1*, no hexokinase; *Curve 2*, 25 microliters hexokinase per vessel. Substrate, L-glutamate (15 m*M*). Time of incubation, 20 min.

Fig. 4 (*right*). Respiration after preincubation with varying concentrations of Ca^{++}. Standard suspension of mitochondria, containing AMP (1.5 m*M*), hexokinase (25 microliters per vessel), Mg^{++} (4 m*M*), and Ca^{++} as indicated. Time of preincubation, 5 min. Substrate L-glutamate (15 m*M*).

This possibility was not incompatible with the observation, made in Table I, that the loss of DPN can reach an advanced stage while the phosphorylative efficiency is relatively intact, if assuming that the two effects may become manifest at different levels of mitochondrial ATP.

In the following sections data supporting this line of reasoning will be presented.

Influence of various factors on the stability of DPN-linked oxidations.—The effect of varying Ca^{++} concentrations is shown in Fig. 1. The inhibition of the respiration appears as a decline, rather than an overall depression, of the respiratory rate, its extent thus being a resultant of Ca^{++} concentration and time of incubation. If hexokinase–glucose is replaced by AMP as the

terminal phosphate acceptor (Fig. 2) the system shows an increased resistance to Ca^{++}. In this experiment, two series of samples, containing varying concentrations of Ca^{++}, were supplemented with identical concentrations of AMP and to one of them hexokinase–glucose was added; a third series (not shown in the figure), with heat-inactivated hexokinase served as a control, in order to rule out the possibility that the observed effect was due to Ca^{++} or other inorganic contaminant in the enzyme preparation. The only respect, thus, in which the two systems essentially differ is that in the AMP series there is an accumulation of ATP during incubation, whereas in the presence of hexokinase ATP is continuously removed from the medium. These results suggest that the progressive character of the Ca^{++} effect, observed in Fig. 1, may be due to a gradual removal of ATP.

TABLE II

Influence of preincubation on the effect of Ca^{++} on respiration.

Standard suspension of mitochondria, containing AMP (1.5 m*M*), hexokinase (25 microliters per vessel), Mg^{++} (4 m*M*) and, where indicated, Ca^{++} (0.125 m*M*). Substrate, L-glutamate. Time of test incubation, 20 min.

Conditions	Ca^{++} absent Oxygen uptake microatoms	Ca^{++} present Oxygen uptake microatoms
Substrate added at 0-time	16.4	15.6
Substrate added after 5 min. of preincubation	15.0	5.5

A further indication of this is given by the fact that the Ca^{++} effect becomes markedly enhanced if the system containing Ca^{++} is exposed to a short preincubation prior to the addition of substrate. The effect of preincubation is illustrated by the experiment in Table II. Of two samples, containing identical concentrations of Ca^{++}, one was supplemented with substrate at 0 time, while the other was preincubated for 5 minutes prior to the addition of substrate from the side arm. At the low Ca^{++} concentration used in this experiment (0.125 m*M*) there is hardly any depression of the respiration (over a period of 15 minutes) in the sample where substrate has been added simultaneously with Ca^{++}; in the preincubated sample, on the other hand, there is a marked inhibition, suggesting that the absence of an active phosphorylation promotes the manifestation of the Ca^{++} effect. Under these conditions, in contrast to those applied in Fig. 1, the respiratory rate is virtually constant and proportional to the concentration of Ca^{++} (Fig. 3).

TABLE III

Influence of Mg^{++} and Mn^{++} and of preincubation time on the effect of Ca^{++} on respiration.

Standard suspensions of mitochondria, containing AMP (1.5 m*M*), hexokinase (25 microliters per vessel), and Ca^{++}, Mg^{++} and Mn^{++}, in concentrations indicated in the table. Time of preincubation, as indicated. Time of test incubation: *Expt. 1*, 20 min.; *Expt. 2*, 15 min. Substrate, L-glutamate. In *Expt. 2*, last series, 2 m*M* Mg^{++} was added in the test incubation.

Expt. No.	Time of preincubation min.	Ca^{++}, m*M*		0	0.25	0.5
		Mg^{++} m*M*	Mn^{++} m*M*	Oxygen uptake microatoms	Oxygen uptake microatoms	Oxygen uptake microatoms
1	0	4		16.4		
	5	8		16.4	11.2	6.5
		4		15.0	5.3	3.2
		2		14.4	2.8	0.0
		2	0.6	16.8	10.7	3.7
2	0	8		13.5		
	10	8		13.0		3.3
	20	8		7.8		0.0
	0	4		12.6		
	10	4		12.4		0.0
	20	4		5.4		0.0
	0	2		12.8		
	10	2		3.9		0.0
	20	2		1.5		0.0
	0	0		12.3		
	10	0		9.4		0.0
	20	0		0.6		0.0

In the preceding experiments the concentration of Mg^{++} has been constant. In Table III some experiments are shown, demonstrating that the inhibition of respiration induced by Ca^{++} during preincubation is a function of the concentration ratio Ca^{++}/Mg^{++}. These experiments contained three variables: Ca^{++} concentration, Mg^{++} concentration, and time of preincubation; in one series, Mn^{++} was added, in order to compare its antagonistic efficiency with that of Mg^{++}. Samples containing the desired cation concentrations were preincubated in Warburg vessels for various periods of time, after which glutamate was added from the side arm, and oxygen uptake recorded during 15 minutes. The data show that the Ca^{++} effect stands in inverse relation to the concentration of Mg^{++}. At low Mg^{++} concentrations, or in the absence of Mg^{++}, prolonged preincubation leads to an inactivation even

without added Ca^{++}, probably due to the presence of endogenous Ca^{++}. It may be the case (Expt. 2), on the other hand, that a low Mg^{++} concentration is more deleterious than no Mg^{++}; in the latter case, as shown previously [13], the lowered activity of hexokinase may constitute a limiting factor for the removal of the ATP released from the mitochondria.

A comparison of the effects of Mg^{++} and Mn^{++} (Expt. 1) reveals that the latter ion is more efficient by a factor of 5 to 10. This value is in agreement with recent data by Hunter and Ford [23]; on the other hand, it is lower than that observed previously [13] in connection with protection of oxidative phosphorylation, where a factor of 50 or more could be established. The reason for this discrepancy is not yet understood.

Effect of hexokinase on respiration in the presence of dinitrophenol.—In the following experiments it was of interest to find conditions under which the depression of the oxidative capacity, taking place during preincubation, could be correlated with a release of mitochondrial ATP in a more conclusive way. It has been observed by Lardy and Wellman [30] and by Siekevitz and Potter [41] that the combined addition of dinitrophenol and hexokinase may cause a decline of the respiratory rate in a mitochondrial system which respires at full capacity in the presence of either of these additions alone. This effect, which has been attributed by the former authors to a lack of ATP in the medium, has been found [11] to be prevented by Mn^{++}, indicating that it is not primarily connected with the action of dinitrophenol on the phosphorylative mechanism; the latter cannot be prevented by Mn^{++}, as also emerges from Table I.

In view of the data obtained in Table III, it was visualized that the dinitrophenol–hexokinase system is, from the point of view of ATP supply, equivalent to a system which is preincubated without substrate. Thus, under these conditions, the actual rate of respiration at any given time may provide a direct measure of the respiratory capacity present in the system after a corresponding time of preincubation. In this way, the decrease of the respiratory capacity during preincubation can be followed as a function of hexokinase concentration, that is, as a function of the rate at which intramitochondrial ATP is removed. In Table IV, some typical experiments are shown. In these experiments, mitochondria were supplemented with AMP, 0.2 m*M* dinitrophenol and 2 m*M* Mg^{++} (at this Mg^{++} concentration, as seen in Table III, a relatively rapid inactivation takes place even without added Ca^{++}), and incubated in the presence of glutamate. Expt. 1 shows that, in the presence of hexokinase, a gradual decline of the respiratory rate ensues during incubation, and that this decline is more marked the higher is the

level of hexokinase. It is noteworthy that the amount of hexokinase needed to accomplish this effect is very high (0.05 ml hexokinase corresponds, according to Fig. 1, to an "excess" of 10 times), which is reasonable if one considers that the concentration of the ATP originating from the mito-

TABLE IV

Effect of hexokinase on respiration in presence of 2,4-dinitrophenol.

Standard suspensions of mitochondria, containing AMP (1.5 m*M*) and Mg^{++} (2 m*M*). Additions (where indicated): 2,4-dinitrophenol (0.2 m*M*), hexokinase (as indicated, per vessel), DPN (0.8 m*M*), Mn^{++} (0.5 m*M*). Substrate, L-glutamate (15 m*M*), added to all samples at 0-time.

Expt. No.	Additions	Interval min.	No dinitrophenol Oxygen uptake microatoms	Dinitrophenol Oxygen uptake microatoms
1	none	5–10	0.5	4.3
		10–15	0.9	4.4
		15–20	0.9	4.1
	0.025 ml hexokinase	5–10	3.9	3.6
		10–15	4.2	4.0
		15–20	3.8	3.2
	0.05 ml hexokinase	5–10	4.3	4.4
		10–15	4.4	2.6
		15–20	4.4	1.3
	0.1 ml hexokinase	5–10	4.1	1.9
		10–15	4.5	1.5
		15–20	4.5	1.0
2	none	7–17	3.9	9.8
	0.1 ml hexokinase	7–17	8.1	2.4
	DPN (added from side arm at 17 min.)	17–27		6.7
	0.1 ml hexokinase, Mn^{++}	7–17		9.1

chondria must be of an extremely low order of magnitude; this explains why the dinitrophenol induced ATP-ase is not sufficient to bring about this effect [25]. A control series without dinitrophenol shows that hexokinase alone has no deleterious effect on respiration within the concentration range and time interval tested. In Expt. 2 it is shown that the decline of respiration occurring in the presence of dinitrophenol plus hexokinase is prevented by Mn^{++} and restored by DPN. These experiments very strongly support the conclusion that the release of mitochondrial DPN following preincubation is a secondary consequence of a release of mitochondrial ATP.

Influence of various factors on stability of DPN-linked oxidative phosphorylation.—In the subsequent experiments the labilization of the phosphorylative ability was followed in relation to the release of DPN. For this purpose, mitochondrial systems were brought to a state of DPN deficiency under various experimental conditions and tested with respect to phosphorylative efficiency in the presence of added DPN. A typical experiment is shown in Table V. In this experiment, mitochondria have been preincubated for 10

TABLE V

Influence of Mg^{++} concentration on re-appearance of phosphorylation in connection with DPN-restored respiration.

Standard suspension of mitochondria, containing AMP (1.5 m*M*), hexokinase (25 microliters per vessel), and Ca^{++} and Mg^{++} as indicated. Time of preincubation, 10 min. Time of test incubation, 20 min. Substrate, L-glutamate (15 m*M*).

Ca^{++}, m*M* added to preincubation	Mg^{++}, m*M* added to preincubation	DPN, m*M* added to test incubation	Oxygen uptake microatoms	Phosphate uptake micromoles
	4		15.6	41.7
	4	0.8	16.1	40.0
0.5	4		0.0	0.0
0.5	4	0.8	12.0	2.8
0.5	6		0.0	0.0
0.5	6	0.8	14.5	24.3
0.5	8		3.0	4.9
0.5	8	0.8	16.0	33.1

minutes in the presence of AMP, hexokinase–glucose, 0.5 m*M* Ca^{++} and varying concentrations of Mg^{++}. At all Mg^{++} levels, preincubation with Ca^{++} resulted in complete or almost complete loss of the oxidative activity. On addition of DPN respiration is restored, but the phosphorylative efficiency is a function of the Mg^{++} concentration present during preincubation. Thus, at 4 m*M* Mg^{++}, phosphate uptake is very low, while at 8 m*M* Mg^{++} the rate of phosphorylation is close to the level obtained in the Ca^{++}-free control.

Further variables involved in the labilization of the phosphorylative system emerge from the experiments diagrammed in Fig. 5. In each experiment two series of samples were preincubated in the presence of varying concentrations of Mg^{++}; in the first experiment (diagram to the left) one series

was preincubated without, and one with 0.5 mM Ca^{++}; both were supplemented with 20 microliters of hexokinase and the time of preincubation was 20 minutes. In the second experiment (middle diagram) both series were preincubated in the presence of 0.5 mM Ca^{++} and 20 microliters of hexokinase, but the one for 5, and the other for 20 minutes. In the third experiment (diagram to the right) Ca^{++} concentration (0.5 mM) and preincubation time (5 minutes) were kept constant and the amount of hexokinase added

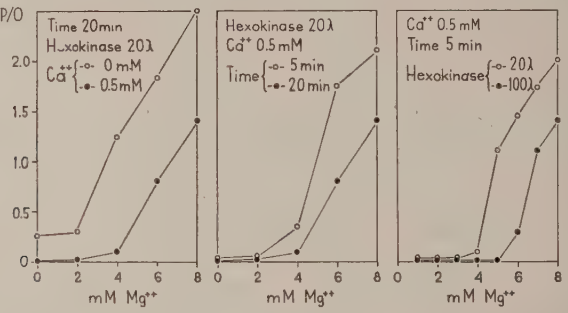

Fig. 5. Effect of preincubating conditions on re-appearance of phosphorylation in connection with DPN-restored respiration. Standard suspensions of mitochondria, containing AMP (1.5 mM), and Ca^{++}, Mg^{++} and hexokinase in concentrations indicated in the individual diagrams. Time of preincubation, as indicated. Test incubations performed in the presence of L-glutamate (15 mM) and DPN (0.8 mM). Time of test incubation, 20 min.

to the preincubating medium was varied (20 and 100 microliters, respectively). In all cases the preincubating conditions were chosen so as to yield a virtually complete depression of the respiratory ability. After preincubation, all samples were supplemented with glutamate and DPN, and oxygen and phosphate uptakes measured over a period of 15 minutes. The results, expressed in terms of P/O ratio, show that the more radical the preincubating conditions are with respect to ATP-depleting power, the lower is the phosphorylative efficiency in the DPN-restored system. These data are thus consistent with the previous assumption that the release of DPN and the loss of the phosphorylative ability are due to a common action, i.e., a lowering of the mitochondrial ATP level, but the two effects become manifest at different levels.

Restoration of phosphorylative ability.—In previous studies with the mitochondrial succinic oxidase system [13] it has been shown that the loss of the phosphorylative ability, caused by pretreatment of mitochondria leading

to a loss of ATP, can be restored by the addition of ATP to the inactivated system, and that this effect of ATP is greatly potentiated by Mn^{++}. The confusing fact that in the DPN linked systems DPN may be lost prior to the loss of the phosphorylative ability can give the impression that in these

TABLE VI

Restoration of phosphorylation coupled to the aerobic oxidation of glutamate.

Expts. 1 and *2*: standard suspensions of mitochondria, containing AMP (1.5 m*M*), hexokinase (25 microliters per vessel), Ca^{++} (0.6 m*M*) and Mg^{++} (4 m*M*). Time of preincubation, 5 min. *Expts. 3* and *4*: standard suspensions of mitochondria, containing AMP and hexokinase as above, but no Ca^{++} or Mg^{++}. Time of preincubation, 15 min.

Additions to test incubation: *All samples:* L-glutamate (15 m*M*); *Expts. 3* and *4*: Mg^{++} (2 m*M*); *where indicated:* DPN (0.8 m*M*), cytochrome *c* (0.01 m*M*), Mn^{++} (0.5 m*M*), ATP (0.8 m*M* or as indicated).

Time of test incubation: *Extpts. 1*, *2* and *4*, 15 min.; *Expt. 3*, 20 min.

Expt. No.	System	Additions to test incubation	Oxygen uptake microatoms	P/O
1	Intact	none	10.9	2.11
	Preincubated	none	1.4	1.64
		DPN	10.2	0.28
		DPN, Mn^{++}, ATP	11.9	1.83
2*	Intact	none	13.0	2.29
	Preincubated	none	2.3	0.70
		DPN	13.3	0.19
		DPN, Mn^{++}, ATP	13.7	1.80
3	Intact	none	15.8	2.80
	Preincubated	none	3.6	0.99
		DPN	14.5	0.35
		DPN, cyt. *c*	16.0	0.39
		DPN, Mn^{++}, ATP	11.6	1.84
		DPN, cyt. *c*, Mn^{++}, ATP	15.8	1.73
4	Intact	none	12.5	2.85
	Preincubated	none	3.0	1.27
		DPN, cyt. *c*	12.8	0.35
		Mn^{++}, ATP (0.8 m*M*)	2.7	2.44
		DPN, cyt. *c*, Mn^{++}	10.0	0.89
		DPN, cyt. *c*, ATP (0.8 m*M*)	10.8	0.42
		DPN, cyt. c, ATP (4.0 m*M*)	7.5	1.20
		DPN, cyt. *c*, Mn^{++}, ATP (0.8 m*M*)	8.8	1.95
		DPN, cyt. *c*, Mn^{++}, ATP (4.0 m*M*)	8 3	1.80

* Mitochondria prepared in the presence of 0.001*M* versene.

systems the addition of DPN alone may be sufficient to restore the phosphorylative ability. In the following experiments data are shown illustrative of the effects of ATP and Mn^{++} under conditions where added DPN alone no longer elicits a phosphorylation. Some typical experiments are shown in Table VI. In Expts. 1 and 2, preincubation was performed in the presence of 0.6 m*M* Ca^{++} and 4 m*M* Mg^{++} for a period of 5 minutes; in Expts. 3

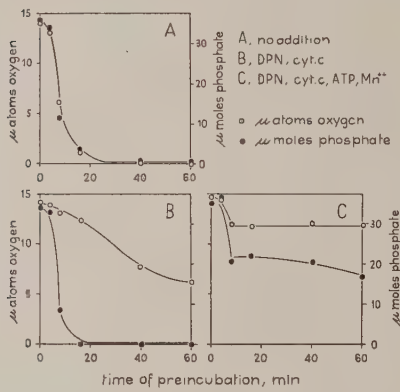

Fig. 6. Restoration of glutamate oxidation and coupled phosphorylation after varying times of incubation. Standard suspension of mitochondria, containing AMP (1.5 m*M*) and hexokinase (25 microliters per vessel). Preincubation without Mg^{++} and Ca^{++}. Additions to test incubations: L-glutamate (15 m*M*), Mg^{++} (2 m*M*) and *A*, none; *B*, DPN (0.8 m*M*), cytochrome *c* (0.01 m*M*); *C*, DPN (0.8 m*M*), cytochrome *c* (0.01 m*M*), ATP (0.8 m*M*), Mn^{++} (0.5 m*M*). Time of test incubation, 15 min.

and 4, the mitochondria were preincubated for 15 minutes in the absence of Ca^{++} and Mg^{++}. These latter conditions are specially suited for studies of restoration of phosphorylative ability, since in the absence of Mg^{++} the phosphorylative ability decreases very rapidly (provided that a large excess of hexokinase is present) and is lost almost simultaneously with the release of DPN (cf. Fig. 6). In all four experiments in Table V, the restoration of respiration by DPN involves little or no phosphorylation, while, in all cases, the addition of ATP and Mn^{++} markedly elevates the rate of phosphorylation. A certain effect may be obtained with Mn^{++} alone, or with ATP alone if this is added in a relatively high concentration (Expt. 4). The data are analogous to those earlier obtained in the succinic oxidase system [13]. Cytochrome *c*, which slightly stimulates DPN-restored respiration, has a parallel effect on phosphorylation, the P/O ratio being essentially unaltered (Expt. 3).

In Fig. 6, the yield of the restored phosphorylation is studied as a function of preincubation time. The three diagrams represent (*A*) the aged system tested with glutamate alone; (*B*) with glutamate, DPN and cytochrome *c*; and (*C*) with glutamate, DPN, cytochrome *c*, ATP and Mn^{++}. As seen, the level of the restored phosphorylation is virtually independent of the time of pre-

incubation within the time range represented by the experiment; if the aging is prolonged beyond 60 minutes a fall may occur, similarly to the case in the succinic oxidase system [13]. The P/O values that can be deduced from the figure are about two-thirds of that in the intact system, but occasionally even higher values, definitely exceeding 2 [33], could be recorded. The relatively high incidence of recoveries of two-thirds in the present system (cf. also Table VI) and of one-half in the succinic oxidase [12, 13] gives rise to the speculation that the restoration of the common phosphorylative step in the two systems—that occurring in the cytochrome region—might require special measures, in addition to the structural reconstruction induced by ATP and Mn^{++}. The possible involvement of a thermostable co-factor, indicated by preliminary studies [14, 15], is a subject of future investigation.

It is noteworthy in Fig. 6 that the level of DPN-cytochrome *c*-restored respiration decreases upon prolonged preincubation (diagram B), whereas in the presence of ATP and Mn^{++} it is maintained constant. This phenomenon will be elaborated on in greater detail in the subsequent paper [10].

Finally, in Table VII, data are shown indicating the principal validity of the above findings for a series of DPN-linked systems other than glutamate. The substrates tested were pyruvate, α-ketoglutarate, citrate, malate and β-hydroxybutyrate, in all cases added to a mitochondrial system previously preincubated for 30 minutes in a Ca^{++}- and Mg^{++}-free medium. DPN and cytochrome *c*, when added alone, restore respiration almost completely in the cases of pyruvate, α-ketoglutarate and β-hydroxybutyrate, but only partially with citrate and malate; the slight phosphorylation appearing in the α-ketoglutaric system probably originates from the succinyl-CoA-linked phosphorylative step. In the presence of ATP and Mn^{++}, the respiratory rates are almost identical with those in the intact system; the phosphorylation recoveries are within the range observed with glutamate, in some cases possibly somewhat lower.

It is interesting to notice the fact that the addition of DPN and cytochrome *c* is sufficient to restore respiration even in the case of the two α-keto acids, indicating that the aging did not lead to a loss of CoA, or at least not to a limiting extent. Brenner-Holzach and Raaflaub [4] have recently reported data revealing a loss of mitochondrial CoA upon aging, but it may be that in the present case this loss had not yet reached a critical level. The interest of these data lies in the fact that DPN and CoA, two molecules of the same order of magnitude, apparently are not lost simultaneously, which reasonably ought to be the case, were their loss due to the loosening of a common permeability barrier.

TABLE VII

Restoration of oxidative phosphorylation in various DPN-linked systems.

Standard suspension of mitochondria, containing AMP (1.5 m*M*) and hexokinase (25 microliters per vessel). Time of preincubation, 15 min. Additions to test incubation: substrate (15 m*M*), Mg^{++} (2 m*M*), and, where indicated, DPN (0.8 m*M*), cytochrome *c* (0.01 m*M*), ATP (0.8 m*M*), Mn^{++} (0.5 m*M*). Time of test incubation, 15 min.

System	Intact		Preincubated					
Additions	None		None		DPN, cyt. *c*		DPN, cyt. *c*, ATP, Mn^{++}	
Substrate	Oxygen uptake microatoms	Phosphate uptake micromoles	Oxygen uptake microatoms	Phosphate uptake micromoles	Oxygen uptake microatoms	Phosphate uptake micromoles	Oxygen uptake microatoms	Phosphate uptake micromoles
Pyruvate	6.9	18.5	0.0	2.1	5.5	1.9	6.1	10.8
Citrate	10.5	21.0	1.0	2.5	3.6	1.9	10.7	12.0
α-Ketoglutarate	11.9	28.4	0.4	2.3	10.9	4.2	12.9	19.8
L-Malate	5.9	16.9	0.3	2.4	3.1	1.8	4.8	6.0
DL-β-Hydroxybutyrate	6.5	18.5	0.0	2.1	5.5	1.9	6.1	10.8

SUMMARY

Ca^{++} inhibits the oxidation of DPN-linked substrates in KCl–sucrose suspensions of rat liver mitochondria incubated in the presence of ATP, orthophosphate, hexokinase, glucose and Mg^{++}. The inhibition appears as a progressive decrease of the respiratory rate, which is more marked the higher the concentration of Ca^{++}.

The extent of the Ca^{++}-induced inhibition of respiration can be reduced by replacing hexokinase-glucose by AMP as the terminal phosphate acceptor; and, conversely, it can be increased if the system is preincubated for a period of time in the absence of oxidizable substrate. The respiratory rate obtained after a given time of preincubation is virtually constant, and proportional to the concentration ratio Mg^{++}/Ca^{++} in the preincubating medium. Mn^{++} exhibits a Ca^{++} antagonism which is about 5–10 times stronger than that of Mg^{++}.

Preincubation with no or little Mg^{++} results in a relatively rapid decrease of the respiratory capacity even in the absence of added Ca^{++}. If substrate is added at 0-time, but phosphorylation is suppressed by 2,4-dinitrophenol, respiration may show a progressive decline, which is proportional to the amount of the hexokinase added to the system.

Under all the above conditions respiration can be restored by DPN, although its rate may be below the original; cytochrome *c* may exhibit a certain enhancement. The DPN restored respiration may or may not be accompanied by a phosphorylation, depending on the concentration ratio of Mg^{++}/Ca^{++} and the hexokinase activity present in the preincubation medium, and on the duration of the preincubation.

When no phosphorylation appears in connection with DPN-restored respiration, the phosphorylative ability can be restored by the addition of ATP; the effect of ATP is greatly potentiated by Mn^{++}. The rate of the restored phosphorylation is generally lower than that in the intact system, the P/O ratio being somewhat below 2.

The data support the conclusion that the release of the mitochondrial complement of DPN and the loss of the phosphorylative ability, following Ca^{++} treatment or preincubation with no or little Mg^{++}, are both secondary consequences of a release of mitochondrial ATP. DPN is lost more readily than is the phosphorylative ability and, thus, addition of DPN to a system, which has not yet reached the stage of lost phosphorylative ability, results in a reappearance of a phosphorylation in connection with the restored

respiration. At such a stage no ATP or Mn^{++} is required for inducing phosphorylation; these requirements appear after pretreatment implying an actual loss of the phosphorylative ability.

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ORGANIZATION OF MITOCHONDRIAL DPN-LINKED SYSTEMS[1]

II. REGULATION OF ALTERNATIVE ELECTRON TRANSFER PATHWAYS

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It has been concluded in Part I of this series [6] that alterations of the mitochondrial organization, elicited by factors inducing a release of mitochondrial ATP, may be manifested in a loss of bound DPN and in a disconnection of the coupling of phosphorylation to electron transport. It has been shown, furthermore, that the latter effect could be reversed by supplementing the uncoupled system with suitable levels of ATP and Mn^{++}. The present paper contains data indicating that the reconstruction of the mitochondrial structure by ATP and Mn^{++} also involves a re-establishment of the functional separation existing in the intact mitochondrion between the organized electron transfer system and external DPN. The bearing of these phenomena on the functioning of alternative electron transfer pathways has been investigated in some detail, using amytal and antimycin A as selective inhibitors. The evidence obtained suggests that DPNH is more firmly associated with the mitochondrial structure than is DPN. The results will be summarized in a hypothetical scheme of the functional pattern of mitochondrial DPN-linked oxidations.

EXPERIMENTAL

Standard suspensions of mitochondria were prepared as follows: Mitochondria from one rat liver (30–40 mg protein-N) were suspended in 20 ml of a solution containing 174 mg K_2HPO_4, 230 mg KCl, 216 mg glucose, 20 mg AMP and 6 mg cytochrome *c*. The suspension was supplemented with 0.5 ml of a stock solution of hexokinase (of a strength defined in Part I, Fig. 1 [6]) and with $MgCl_2$ and $CaCl_2$ in concentrations specified in the individual experiments, and diluted with 0.25*M* sucrose to a final volume of 30 ml.

[1] *Abbreviations:* AMP, adenosine-5-phosphate; ATP, adenosine triphosphate; DPN, diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide.

Preincubations were performed as described previously [6]. For test incubation, 1.5 ml aliquots of the standard suspension were used, diluted, by adding substrate and the desired individual additions, to a final volume of 2 ml.

Crystalline yeast alcohol dehydrogenase and crystalline liver glutamic dehydrogenase were obtained from Boehringer, Mannheim, Germany. Amytal (5-ethyl-5-*iso*amylbarbituric acid) was a common pharmacy product. Solutions of amytal were prepared as described elsewhere [8]. A preparation of antimycin A was kindly supplied by Dr. B. C. Pressman, Madison, Wisconsin. 1 mg antimycin A was dissolved in 5 ml *iso*propanol and diluted with water to 10 ml (25). 10 microliters of this solution were used per Warburg vessel.

For further experimental details see Part I [6].

RESULTS

It has been shown previously [17, 10, 8] that narcotically active barbiturates, e.g. amytal, block mitochondrial DPN-linked oxidations, but the inhibition is only partial if DPNH is supplied from an external source to the mitochondria. With glutamate as a substrate, for example, a certain respiration could be obtained in the presence of amytal, if the system was supplemented with crystalline glutamic dehydrogenase; this respiration, in contrast to that exhibited by the original, uninhibited system, was dependent on added DPN and cytochrome *c*, and it was not accompanied by an uptake of phosphate. According to current concept, this type of DPNH oxidation is due to an "external", nonphosphorylative form of DPNH-cytochrome *c* reductase, consistently present in mitochondrial preparations, but located outside the organized mitochondrial structure, and differing from its "internal" counterpart in being insensitive to the specific inhibitors, amytal and antimycin A [20, 21, 30, 5, 8].

The fact that no significant respiration could be obtained in the amytal-containing system even in the presence of added DPN and cytochrome *c*, unless an external dehydrogenase was added, has been interpreted to indicate that, in the intact mitochondria, there is some kind of a structural barrier which restrains the capacity of the internal dehydrogenases to produce external DPNH [8]. As shown in Table I, this situation is altered if the mitochondria are exposed to a pretreatment leading to a loss of their stock of DPN. In such a system, the addition of DPN results (in the presence of added cytochrome *c*) in a respiration which is but partially sensitive to amytal or antimycin A, even when no external dehydrogenase is added. The amytal- and antimycin A-insensitive respiration can be depressed close to the level prevailing in the intact system by treatment with ATP and Mn^{++} (Table I, Expts. 2 and 3).

TABLE I

Effect of pretreatment on amytal- and antimycin A-sensitivity of respiration.

Standard suspensions of mitochondria (cf. Experimental). Pretreatment: *Expts. 1* and *2:* 15 min., without Ca^{++} or Mg^{++}; *Expt. 3*: 5 min., with 1.0 m*M* Ca^{++} and 4 m*M* Mg^{++}. Additions to test incubation: *all samples:* L-glutamate (15 m*M*); *Expts. 1* and *2:* Mg^{++} (2 m*M*); *where indicated:* DPN (0.8 m*M*), glutamic dehydrogenase (ca. 0.1 mg per vessel), Mn^{++} (0.5 m*M*), amytal (1.8 m*M*), antimycin A (1 microgram per vessel). Time of test incubation: *Expt. 1*, 10 min.; *Expts. 2* and *3*, 20 min.

Expt. No.	System	Additions to test incubation	No inhibitor Oxygen uptake microatoms	Amytal Oxygen uptake microatoms	Antimycin A Oxygen uptake microatoms
1	Intact	none	9.9	0.0	
		DPN	10.5	0.8	
		DPN, glutamic dehydrogenase		4.2	
	Pretreated	none	2.5	0.0	
		DPN	11.1	3.9	
2	Intact	none	18.5	0.0	0.0
		DPN	20.7	1.6	1.8
	Pretreated	none	5.3	0.0	0.0
		DPN	16.3	7.0	6.5
		DPN, Mn^{++}, ATP (0.8 m*M*)	16.0	3.9	2.5
3	Intact	none	17.3		
		DPN		1.4	
	Pretreated	none	0.0	0.0	
		DPN	5.3	5.3	
		DPN, Mn^{++}	7.1	5.7	
		DPN, Mn^{++}, ATP (0.8 m*M*)	8.9	3.4	
		DPN, Mn^{++}, ATP (1.6 m*M*)	10.5	2.5	
		DPN, Mn^{++}, ATP (2.4 m*M*)	10.5	1.5	

Of two conceivable explanations that the observed phenomena were due (1) to a reversible insensitization of the internal DPNH-cytochrome *c* reductase to amytal and antimycin A, or (2) to a reversible relief of the above postulated structural barrier between internal dehydrogenases and external DPN, the former appeared improbable, as the succinic-cytochrome *c* reductase system is sensitive to antimycin A independently of the structural state of the mitochondria [11]. In attempting to test the latter alternative, it was visualized that, if ATP and Mn^{++} act by re-establishing a structural barrier between internal dehydrogenases and external DPN, it should be possible to *prevent* DPN from restoring respiration in a DPN-deficient system,

TABLE II

Prevention of DPN-induced restoration of respiration by treatment with ATP and Mn^{++}.

Standard suspension of mitochondria, containing no Ca^{++} or Mg^{++}. Additions (at indicated times): L-glutamate (15 m*M*), DPN (0.8 m*M*), ATP (0.8 m*M*), Mn^{++} (0.5 m*M*), succinate (15 m*M*). 2 m*M* Mg^{++} added simultaneously with addition of substrate. Time of test incubation, 15 min.

Time of addition min.	Additions	Oxygen uptake microatoms	Phosphate uptake micromoles
0	glutamate	13.2	33.2
15	glutamate	1.4	0.0
15	glutamate, DPN	11.2	3.4
15	glutamate, DPN, ATP, Mn^{++}	10.9	25.0
15	glutamate, ATP, Mn^{++}	4.3	5.2
20	DPN		
0	succinate	12.2	26.6
15	succinate	7.6	0.0
15	succinate, ATP, Mn^{++}	12.3	14.8
15	ATP, Mn^{++}	12.6	14.8
20	succinate		

if the latter is subjected to a short treatment with ATP and Mn^{++} *prior* to the addition of DPN.

An experiment proving this line of reasoning is shown in Table II. The mitochondria were brought to a DPN-deficient state by preincubation for 15 minutes in a Mg^{++}-free medium. After this treatment aliquots were taken out and incubated with the following additions: (1) with glutamate alone, in order to test that DPN is lost; (2) with glutamate and DPN, whereby respiration is restored; (3) with glutamate, DPN, ATP and Mn^{++}, whereby both respiration and phosphorylation are restored, indicating that a structural reconstruction has taken place; and, finally, (4) with glutamate, ATP and Mn^{++}, and, after an interval of five minutes, with DPN. In this case, as seen in Table II, respiration is markedly depressed as compared to the cases (2) and (3), indicating that, after treatment with ATP and Mn^{++}, external DPN no longer has free access to the mitochondrial glutamic dehydrogenase. In a control series, with succinate as a substrate, it could be ascertained that the ATP–Mn^{++}-induced reconstruction actually had lasted for the five minutes in question, and that, consequently, the depression of the respiration, observed in the DPN system, was not due to a secondary —denaturative—consequence of the delay.

TABLE III

Comparison of some effects of preincubation on intra- and extramitochondrial DPNH-generating systems.

Standard suspension of mitochondria containing Mg^{++} (4 m*M*) and Ca^{++} as indicated. Additions to test incubation (where indicated): L-glutamate (15 m*M*), ethanol (15 m*M*), alcohol dehydrogenase (ADH, ca. 0.06 mg per vessel), DPN (0.8 m*M*), ATP (0.8 m*M*), Mn^{++} (0.5 m*M*), amytal (1.8 m*M*). Time of test incubation, 15 min.

Pretreatment with Ca^{++} m*M*	Additions	Glutamate Oxygen uptake microatoms	Ethanol+ADH Oxygen uptake microatoms
0	none	15.6	0.0
	DPN	16.0	8.7
	DPN, amytal	0.4	7.4
0.5	none	1.5	0.0
	DPN	11.9	8.1
	DPN, amytal	2.4	7.5
1.0	none	0.6	
	DPN	8.0	
	DPN, amytal	7.0	

Having thus obtained evidence for the existence of a reversible mechainsm by which the access of external DPN to mitochondrial dehydrogenases can be regulated, it was of interest to investigate how the same principle applied to the entrance of external DPNH to the internal DPNH-cytochrome *c* reductase, a problem earlier examined by Lehninger [20, 21]. In Table III, a comparison is made between the effects of Ca^{++} treatment on the amytal sensitivity of the oxidation of internally and externally generated DPNH. As an internal DPNH source glutamate plus the mitochondrial glutamic dehydrogenase was used, while, in a parallel series, external DPNH was generated by added ethanol plus alcohol dehydrogenase. Both series were incubated in the presence of cytochrome *c*. In the Ca^{++}-free control, the respiration of the two systems differs in two principal respects: the internal system is independent of added DPN and highly sensitive to amytal, in contrast to the external system, which is strictly dependent on added DPN and but slightly inhibited by amytal. When pretreated with 0.5 m*M* Ca^{++}, the internal system becomes DPN-dependent, but still it is markedly more sensitive to amytal than the external one, whose amytal sensitivity is virtually unaltered by the Ca^{++} treatment. At 1.0 m*M* Ca^{++}, the respiration pattern

of the internal system becomes similar in all respects, to that previously found characteristic for the external system.

These data seem to reveal that a free access of the system to external DPN does not necessarily imply a free access to external DPNH; were that the case, the total respiration of the external system would have increased, upon treatment with 0.5 m*M* Ca^{++}, by an amount corresponding to the amytal

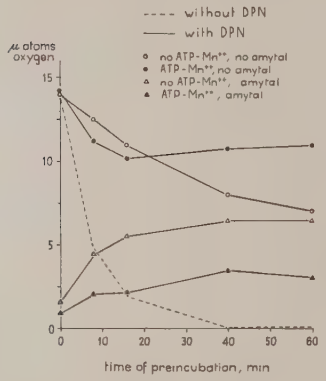

Fig. 1. Effect of aging on amytal sensitivity of DPN-restored respiration. Standard suspension of mitochondria preincubated in the absence of Ca^{++} and Mg^{++}. Additions to test incubation: *All samples:* L-glutamate (15 m*M*), Mg^{++} (2 m*M*); *where indicated:* DPN (0.8 m*M*), ATP (0.8 m*M*), Mn^{++} (0.5 m*M*), amytal (1.8 m*M*). Time of test incubation, 15 min.

sensitive respiration in the internal system, since alcohol dehydrogenase was present in large excess. The high amytal sensitivity of the internal system, observed at this stage of pretreatment, furthermore suggests that the DPNH formed by the internal dehydrogenase still preferentially reacts with the internal reductase, despite the fact that the passage of DPN is free. These results indicate, therefore, that the effect of Ca^{++} cannot be explained in terms of a relief of a structural barrier in the general sense. It would rather appear that DPNH is more firmly associated with the mitochondrial structure than is DPN, and that it is the strength of these associations that determines to what extent the two forms of DPN can gain access to the mitochondrial sites, when supplied from an external source.

On advanced disorganization of the structure—as at 1.0 m*M* Ca^{++} in the above experiment—apparently the ability to bind DPNH is also lost. This may account for the fact, observed previously [6], that on prolonged aging in a Mg^{++}-free medium the DPN-cytochrome *c*-restored respiration, with glutamate as a substrate, decreases. In fact, as shown in Fig. 1, under these conditions it is the amytal-sensitive type of respiration that declines with increasing aging time. It is also seen in Fig. 1 that this effect is reversed by treatment with ATP and Mn^{++}. The reversible release of mito-

chondrial DPNH seems thus to represent a further facet of ATP-dependent structural organization.

The present conclusions stand in line with the recent observation of Hunter *et al.* [15, 16] that under anaerobic conditions mitochondria show an increased resistance to pretreatment entailing a loss of DPN. Under these conditions DPN is conceivably present in the reduced form (due to the

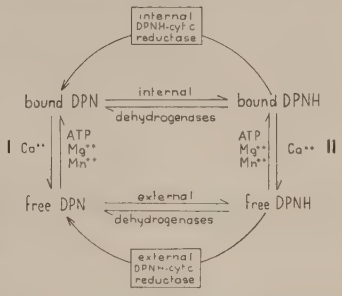

Fig. 2. Representation of the influence of structural alterations on the functional pattern of DPN-linked oxidations. The figures I and II refer to the postulate, made in the text, that bound DPN is more readily dissociated from the structure than is bound DPNH.

presence of small amounts of endogenous substrate) and as such it is more firmly bound to the structure. Pressman [24, 25] recently made the interesting observation that the antimycin A sensitivity of DPN-supplemented mitochondrial systems may vary with the substrate used: it may be high with β-hydroxybutyrate and low with malate. In view of the present explanation this may be due to the fact that the equilibrium of the β-hydroxybutyrate dehydrogenase is strongly shifted towards the formation of DPNH, while that of the malic dehydrogenase favors the oxidized state of DPN. A similar deduction can be made from the data shown in Part I of the present work [6], where, in Table VI, it emerges that, at a given stage of aging, the DPN-cytochrome *c*-restored respiration is high with β-hydroxybutyrate and with the α-keto acids as substrates, while it is low with malate and citrate. In all these cases a high respiration ensues upon treatment with ATP and Mn^{++}. Apparently, the actual capacity of the internal DPNH-cytochrome *c* reductase in a labile mitochondrial system is a function of the DPNH-level that can be provided by the internal dehydrogenases.

This line of reasoning is schematically illustrated in Fig. 2 where the numbers I and II indicate the "order" in which bound DPN and DPNH are released upon advancing labilization of the structure.

The proposed scheme accounts for the fact, observed by Lehninger [20], that the extent to which external DPNH reacts by way of the internal DPNH-cytochrome *c* reductase may be a function of *both* the structural state of the

mitochondria *and* the equilibrium constant of the external dehydrogenase used for generating DPNH. Lehninger found that no phosphorylation could be obtained with ethanol plus alcohol dehydrogenase as a DPNH source, unless the mitochondria were pretreated in a hypotonic medium, while no such pretreatment was necessary if DPNH was generated by the phosphoglyceraldehyde dehydrogenase reaction. It should be added, on the other hand, that under the conditions used by us [8], or by Lardy [19], a certain extent of phosphorylation occurs even in the case of alcohol dehydrogenase-generated DPNH. The extreme tightness of Lehninger's system may be due to the presence of a relatively large concentration of fluoride, which, according to Siekevitz and Potter [31], immobilizes mitochondrial nucleotides.

DISCUSSION

The data obtained in Parts I and II of the present work reveal that a labilization of the mitochondrial structure is reflected in a multiple effect on the organization of the DPN-linked enzyme systems. By applying suitable experimental conditions some of these effects could be separated from each other and evidence could be obtained that each of them is primarily due to a mobilization of mitochondrial ATP. Such conditions could be achieved by exposing mitochondrial suspensions to aging for a given time period in the presence of varying concentrations of Ca^{++} and Mg^{++}, or for varying time intervals in the absence of these ions. In all cases the treatments were performed in the presence of hexokinase and glucose in order to remove the released ATP. The stages of labilization obtained along these variables have been tested with respect to three characteristics: (1) the ability of the system to maintain its stock of DPN, as tested in terms of dependence of respiration on added DPN; (2) the ability to carry out coupled phosphorylation; and (3) the ability to maintain DPNH in the bound state, as deduced from the level of amytal sensitive respiration in the presence of added DPN.

Three stages could thus be distinguished, characterized by (1) strict dependence of respiration on added DPN, relatively intact phosphorylative ability, high amytal sensitivity; (2) strict DPN-dependence of respiration, no phosphorylative ability, high amytal sensitivity; and (3) strict DPN-dependence of respiration, no phosphorylative ability, no amytal sensitive respiration. It has been shown, furthermore, that treatment with ATP and Mn^{++} induces a reconstruction of the system with respect to all three of these characteristics. These results are summarized in a scheme in Fig. 3;

the scheme is somewhat idealized, not taking into account a possible overlapping of the individual stages.

It should be pointed out that this picture is by no means regarded as complete, neither as to the spectrum of enzymic alterations, nor with respect to the involved variables. The dissociation of various nucleotides from the labilized mitochondrial structure seems to follow individual patterns [2, 31];

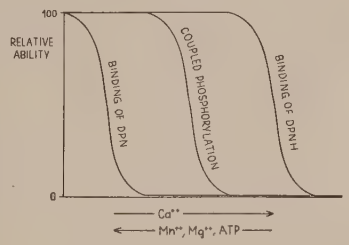

Fig. 3. Scheme illustrating reversible loss of some organized properties of mitochondria, connected with structural labilization.

the relation of these to the mobilization of mitochondrial ATP, and their bearing on organized enzymic activities, are subjects of further studies. Among further agents of biological importance, which appear to influence mitochondrial stability, phosphate [28, 15] and potassium [27] deserve future interest.

Very little can be settled at present as to the mode in which ATP and the bivalent cations act on mitochondrial organization. The possibility has been discussed by several authors [28, 15] that ATP may act as a chelating agent, binding Ca^{++}, and thus protecting some metal prosthetic groups necessary for maintaining the enzymic and osmotic integrity of the mitochondrion. In fact, the protective action of ATP is readily duplicated by versene and a series of other Ca^{++} chelators [28, 15]. However, as has been pointed out previously [9], these agents are not able to duplicate the ability of ATP to even restore the integrity of a previously disorganized system. Although it may very well be that a chelation is ultimately involved in the chemical events underlying the action of ATP—there is hardly any known reaction of ATP in which no bivalent ions are involved—it appears difficult to escape the conclusion that ATP acts on mitochondrial organization in some specific way. The alternative possibility considered by Slater and Cleland [32] that "Ca^{++} might displace some metal prosthetic group from an enzyme which operates in conjunction with $\sim$ P to maintain the morphological and enzymic structure" of the mitochondrion, seems therefore preferable, especially in view of the fact recently reported by Siekevitz and Potter [31] and Beyer

et al. [1] that the terminal phosphate group of intramitochondrial ATP undergoes a rapid turnover in the intact mitochondrion.

According to a recent report by Cooper *et al.* [4] the entire complement of enzymes catalyzing the aerobic oxidation of β-hydroxybutyrate, including the system responsible for coupled phosphorylations, has been successfully isolated from digitonin treated rat liver mitochondria. This organized submitochondrial entity is not sensitive to Ca^{++} and requires no Mg^{++} for exhibiting oxidative phosphorylation. These findings point to the possibility that the high energy level maintained by ATP within the mitochondrial structure is not responsible for the organization of the mitochondrial enzyme system *per se*, but rather for a maintenance of this organization in a labile state of equilibrium.

Alterations of the intramitochondrial energy level, taking place according to the reversible mechanism outlined in the present work, may thus provide a flexibility to the energy transfer system, involving a regulation of the functional coupling between the individual enzymic principles.

According to a theory advanced by Lardy [18] certain hormones may regulate metabolic rates by specifically uncoupling one rate limiting phosphorylative step from electron transport, with a net increase of the rate of output of high energy phosphate as a consequence; the theory presupposes a constantly stable state of the mitochondrial structure, implying an unconditional dependence of respiration on the availability of inorganic phosphate and phosphate acceptors. The data obtained in the present work suggest an alternative hypothesis, based on a reversible labilization of the mitochondrial structure as a physiological control principle. Recent development of the thyroid hormone action appears to lend support to this hypothesis. Thus, mitochondria prepared from thyrotoxic animals, or treated with thyroid hormones *in vitro*, may reveal, besides of a lowered phosphorylative efficiency, also a deficient complement of DPN [22, 14]. These effects can be prevented *in vitro* by Mn^{++} or Mg^{++} [23]. Thyroxin accelerates, furthermore, the swelling of isolated mitochondria [33], at the same time as it is without effect on coupled phosphorylations in the submitochondrial particles described by Cooper *et al.* [4]. These findings indicate that the action of thyroid hormones on oxidative phosphorylation is due to a general labilization of the mitochondrial structure rather than to a true, dinitrophenol-like uncoupling effect.

It is not possible to decide as yet whether the present mechanism can account for a stepwise disconnection of the individual phosphorylations, in the sense visualized by Lardy [18]. An attractive possibility lies, however,

in the fact that a shunting of the oxidation of mitochondrial DPNH over the non-phosphorylative pathway of DPNH-cytochrome *c* reductase may involve, at least theoretically, the by-passing of two rate limiting [3] phosphorylative steps and, thus, an acceleration of the net rate of phosphorylation.

A labilization of the DPN site of the mitochondrial respiratory system may also be of significance for the regulation of the *cellular* DPNH/DPN level [7], which, as signalled by recent reports [12, 13], seems to be a sensitive indicator of the capacity of reductive syntheses in a living cell. Although DPNH may readily be available *within the mitochondria* when respiration is blocked by lack of inorganic phosphate or phosphate acceptor [3, 19], it is difficult to visualize how an output of DPNH into the cytoplasm can take place from the tightly concatenated electron transport system present in the stable mitochondrial structure.

SUMMARY

Structural labilization of mitochondria, as induced by pretreatment in the presence of Ca^{++} or in the absence of Mg^{++}, in both cases with hexokinase-glucose in the preincubating medium, results in a decreased sensitivity of respiration to amytal and antimycin A, as tested in the presence of DPN and cytochrome *c*, and with glutamate as a substrate. The amytal and antimycin A-sensitivity of respiration is restored by ATP and Mn^{++}.

If mitochondria, brought to a DPN deficient state by preincubation in the absence of Mg^{++}, are treated for five minutes with ATP and Mn^{++}, they become partially inaccessible to added DPN.

Upon treatment of mitochondria with increasing concentrations of Ca^{++}, or for increasing periods of time in the absence of Mg^{++}, there is an initial disparity between the rates at which external DPN and DPNH gain access to the intramitochondrial enzymic sites. Upon relatively mild treatment, the mitochondrial complement of DPN is lost and external DPN acquires free access to the mitochondrial dehydrogenases; at the same time, external DPNH has significantly lower access to the amytal sensitive DPNH-cytochrome *c* reductase than has internal DPNH. More drastic treatment results in a gradual decrease of the level of the DPN-dependent, amytal-sensitive respiration, but this can be restored by ATP and Mn^{++}.

These results have been interpreted to indicate that DPN and DPNH are reversibly associated with the mitochondrial structure, but the association of DPNH is firmer than that of DPN.

The experimental data presented in Parts I and II of the present work

are discussed in relation to the role played by ATP in maintaining the mitochondrial structure in a reversible state of organization, and to the implications of this mechanism to the functional pattern of the mitochondrial energy transfer system. It is postulated that a reversible labilization of the mitochondrial structure may constitute a physiological principle of metabolic control.

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BRIEF NOTES

GASTRULATION IN THE SEA URCHIN LARVA STUDIED BY AID OF TIME-LAPSE CINEMATOGRAPHY

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The mechanism of gastrulation, notably that of invagination, continues to be an object of great interest. This problem has been subject to thorough descriptive and experimental analysis in the amphibians, where the mechanism appears to be rather complex. A simpler type of mechanism may be expected to occur in many invertebrates. A careful study of the morphology of gastrulation in some representatives from this category of animals is of fundamental importance to future experimental studies.

By means of time-lapse cinematography each morphological change in the embryo can be registered during the process of gastrulation. The transparency of many marine larvae facilitates the use of this technique. However, the rapid swimming movements of this kind of embryos constitute a serious obstacle to time-lapse filming and makes it impossible to keep the embryos in a constant position. This explains why e.g. the sea urchin larva has so far not been thoroughly analyzed as to its post-hatching morphogenetic movements, although the segmentation of the sea urchin egg was studied already at the beginning of this century.

During the past three years the authors worked out a method by means of which the swimming movements are eliminated and the larvae are kept in a constant position for at least 12 hours. When using this method which is especially adapted for sea urchin larvae, the further development of the larvae is not affected.

The device for keeping the larvae in a constant position consists of a piece of nylon net inserted between the slide and the coverglass. The nylon threads were made rough by a precipitate of calcium carbonate and were thus rather similar to barbed wire. The cross section of the meshes was somewhat larger than the diameter of the blastula. When an egg suspension was placed on the net and the coverglass was placed in position, the larvae were individually caught in the meshes. The calcium carbonate crystals caused slight depressions in the walls of the larvae which were thus kept in a constant position without much interference in their ciliar acitivity. A slow current of water was continuously passed below the coverslip by means of filter paper strips, one of which was siphoning water from a cuvette to the right edge of the cover glass, the other from the left edge to an effluent beaker. The morphogenetic movements were registered by time-lapse cinematography with a frequency of one picture per ten or nineteen seconds. The temperature was about 22°C.

An analysis of 350 meter time-lapse film of gastrulating larvae of *Psammechinus miliaris* revealed that there are two main phases in invagination. In the first the

archenteron rudiment elongates to about $^1/_4$ to $^1/_3$ its final length. This phase of invagination is probably autonomous, i.e. essentially dependent on factors residing in the vegetal region itself. Invagination can thus be attained in an isolated vegetal fragment of a blastula [3]. After this phase of invagination the archenteron tip will more or less remain on the same level for some time, or slowly advance in the animal direction. During the second phase of invagination the process occurs at a considerably higher rate than during the first phase and is here caused by pseudopodia emitted by the archenteron tip and attaching themselves to the ectoderm. The archenteron tip is pulled in the animal direction by contractions of these pseudopodia. The role of the pseudopodia is evident from the fact that their appearance closely corresponds to the increased rate of invagination, and also that a break of the pseudopodia can be correlated with a retardation of invagination. Furthermore, a bend of the archenteron is preceded by an eccentric formation of the pseudopodia in the same direction. Sooner or later the pseudopodia forming cells themselves are extracted from the archenteron tip owing to the contractions of the pseudopodia, which may be explained by the fact that the intestine cannot respond to the contractions at a sufficient rate. The cells liberated are now called secondary mesenchyme cells. The connections between these cells and the archenteron tip may be completely or partially interrupted during extraction. In such cases the invagination ceases until new pseudopodia are formed.

A detailed description with further data on the technical device for this study and on results obtained will be presented in a later publication [2]. After our paper [2] had been submitted for publication in this journal, an important paper on the mechanism of invagination [1] appeared. The data presented therein on the contractile elements connecting the archenteron tip with the ectoderm are also in good agreement with our own observations.

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THE METABOLISM OF PENTOSE PHOSPHATE IN SEA URCHIN SPERM AND EGGS

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The mechanism of stepwise oxidation of glucose through the glucose-6-phosphate dehydrogenase shunt has recently been elucidated by the investigations of Racker

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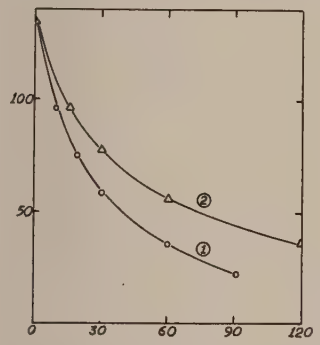
and of Horecker. Several steps of this pathway have been found in a great variety of plants, microorganisms and higher vertebrates (see for ref. [1, 2, 4, 11]).

The existence of an operating phosphorylative oxidation pathway of carbohydrates in sea urchin eggs was first suggested by Runnström [13, 14] and later indicated by the work of Örström and Lindberg [10] and Lindberg and Ernster [8]. A hexokinase which phosphorylates glucose and to a less extent mannose and galactose was found



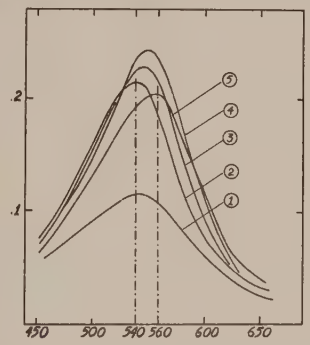


Fig. 1 (*left*). Breakdown of ribose-5-phosphate by cell-free extracts of *Arbacia* sperm (1) and eggs (2) as determined by the orcinol reaction. Abscissa: Min. of incubation. Ordinate: γ of pentose. Fig. 2 (*right*). Formation of ketopentoses and ketohexoses by cell-free extracts of *Arbacia* sperm as indicated by the cystein-carbazole reaction. (1) Zero time; (2) after 15 min.; (3) after 30 min.; (4) after 60 min.; (5) after 90 min. Abscissa: Wavelength. Ordinate: Optical density.

in unfertilized and fertilized eggs by Krahl *et al.* [6]. The same authors have recently studied the dehydrogenation of glucose-6-phosphate and of phosphogluconate by the corresponding TPN depending enzymes in *Arbacia* eggs; they suggest that the glucose-6-phosphate dehydrogenase system may be the major pathway for utilization of carbohydrates in sea urchin eggs [7]. There is no information available concerning the existence of some steps of the glucose-6-phosphate dehydrogenase shunt in sea urchin sperm.

In this note evidence is given for the presence in sea urchin eggs and sperm of an active enzymatic system utilizing pentose-5-phosphate.

Fresh sperm and eggs of *Arbacia lixula* and of *Paracentrotus lividus* were used. The jelly layer of the eggs was removed by treating one volume of egg suspension with one volume of 0.005*N* HCl in sea water. The eggs were homogenized with 0.01*M* phosphate buffer pH 7.2 in a tissue grinder and, after standing for two hours in the cold, the thick paste was centrifuged at 12,000 rpm.

Sperm extracts were prepared by crushing the frozen cells in the press described by Hughes [5]. The paste was diluted with 5 vol. of 0.01*M* phosphate buffer pH 7.2 and extracted in the cold for two hours. The viscous suspension was centrifuged in the cold at 12,000 rpm.

The Ba salt of ribose-5-phosphate was converted into the K salt before use. Total nitrogen in the cell-free extracts was determined in a micro-Kjeldahl apparatus and

TABLE I

Synthesis of fructose from ribose-5-phosphate by cell-free extracts of *Paracentrotus* sperm.

Time min.	μM Ribose-5-phosphate disappeared	μM Fructose formed
0	0	0
15	0.22	0.06
30	0.33	0.13
60	0.42	0.18
90	0.50	0.16

the protein concentration was calculated by using a factor of 6.2. Fructose was determined colorimetrically by the method of Roe [12]. Ketohexoses, ketopentoses and trioses were estimated by the method of Dische and Borenfreund [3]. The presence of ketohexoses and of ketopentoses is indicated by the development of a purple color after a few minutes. Ketohexoses have a maximum absorption at 560 mμ and ketopentoses at 540 mμ. If trioses are present, a blue color with a maximum absorption at 660 mμ develops at the end of 24 hrs. Ribose was determined by the modified orcinol reaction of Mejbaum [9] using 0.033 per cent of $FeCl_3$ and a 30 min. period of heating. The same reaction was used for the detection of sedoheptulose, which has a maximum absorption at 580 mμ, and for ribulose-5-phosphate which has a small peak at 540 mμ together with a large one at 660 mμ, the latter being that of aldo-pentoses. All spectrophotometric measurements were made with a Beckman Model DU spectrophotometer.

Cell-free extracts of sperm and eggs were incubated at 35°C with 10 μM of ribose-5-phosphate in 0.01M phosphate buffer pH 7.2 in presence of 0.05M $MgCl_2$. At intervals samples of the mixture were taken, deproteinized with TCA 10 per cent and analysed for pentoses and hexoses.

Addition of ribose-5-phosphate to cell-free extracts of *Arbacia* and *Paracentrotus* eggs and sperm caused a rapid disappearance of this pentose as shown by the orcinol reaction measured at 660 mμ (Fig. 1). At the same time there was a definite increase in light absorption at 580 mμ, the absorption peak of heptulose, which is an indication of the presence of transketolase. The appearance of ribulose-5-phosphate formed by phosphopentose isomerase was demonstrated by the peak at 540 mμ in the cystein carbazole reaction. The ketopentose appeared after 15 min. and increased in concentration up to 60 min. to be replaced later by fructose as indicated by the shifting of the peak to 560 mμ (Fig. 2).

The quantitative formation of fructose as determined with Roe's reaction is reported in Table I.

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THE RELATION OF DNA CONTENT TO CELL SIZE IN *ASPERGILLUS*

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As is usually the case in other organisms, the DNA content of diploid nuclei of *Aspergillus nidulans* is about twice that of haploid nuclei [3]. Similarly, in *Aspergillus sojae* the same relation holds. In this organism the size of the conidia and the DNA content per conidium are the same in haploids and diploids [4]. The number of nuclei in the diploid conidium is, however, half that in the haploid and thus the constancy of the DNA-cytoplasmic ratio is maintained (Table I).

The strains of *Asp. nidulans*, *Asp. niger* and *Penicillium chrysogenum* studied by Pontecorvo and his coworkers [6, 7, 8] have uninucleate conidia and, consequently, the DNA-cytoplasmic ratio in diploids cannot be maintained, as it is in *Asp. sojae*, by a reduction in nuclear number. Rather, it is maintained by an increase in the amount of cytoplasm and hence in the size of the spores which have about double

Table I

Interrelations between DNA, cell volume and nuclear number in the conidia of haploid and diploid *Asp. sojae*.

Average	Conidial volume (μ3)	DNA per conidium μg ($\times 10^{-7}$)	Number of nuclei per conidium	DNA per nucleus μg ($\times 10^{-7}$)
Haploid[1]	135	3.71	4.22	0.88
Diploid[2]	128	3.73	2.21	1.69

[1] The haploid strains studied were Wild type and Yellow leucineless mutant (y leu).
[2] The diploid strains were Green prototroph (y leu/al his) and Yellow prototrophic segregant (from y leu/al his).

Table II

The relation between cell volume and nuclear number in conidia of the homocaryons and heterocaryons of *Asp. sojae*.

Average	Conidial volume (μ3)	Number of nuclei per conidium	Ratio of cell volume to nuclear number
Homocaryons	138	3.96	35
Heterocaryons	199	5.51	36

The data on heterocaryons come from the cultures y leu + al his; y arg + al leu, and y arg + al his.

The data on homocaryons were secured from component strains grown separately.

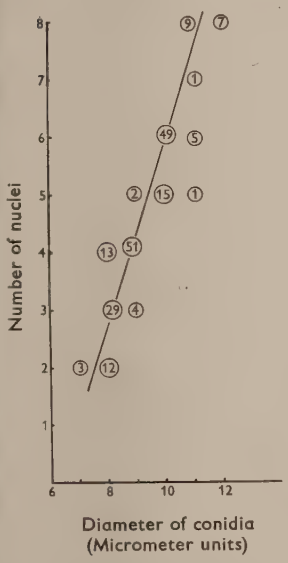
the volume of haploid spores. Thus, fungi may maintain the DNA-cytoplasmic ratio in either of two ways; first, by reducing the number of nuclei when they are diploid and contain more DNA, and, second, by increasing the amount of cytoplasm when the diploid nuclei are single.

In *Asp. sojae* the second method seems also to be used in heterocaryons where the average number of haploid nuclei per conidium is greater than in homocaryons, and where the conidial volume is correspondingly increased (Table II). The number of contained nuclei is directly proportional to the conidial volume (Fig. 1). In collecting the data on heterocaryotic conidia an attempt was made to measure only conidia that came from heterocaryotic conidiophores. Conidial samples from heterocaryotic cultures were found to contain many spores (ca. 33 per cent) where, in addition to the nuclei, the cell walls stained heavily with basic fuchsin when applied by the method of Delamater [1, 2]. Such spores were not found in samples from cultures of homocaryons [5]. On the presumption that these unusual spores were formed on heterocaryotic conidiophores, they were selected for measurement and provide the data in Table II. It has been shown that the size of conidia does not change upon fixation and staining [7].

Their large size (Table II) is further evidence of their heterocaryotic origin as can be seen from Fig. 2. A sample of spores from heterocaryons has a class with a size distribution like that of homocaryons and also a larger class. The staining class is this large class and shows both of the unique properties of conidia found in samples from heterocaryons. In support of the assumption that the heavily staining spores were heterocaryotic is the following additional evidence. When conidia from a heterocaryotic culture grown on minimal medium were plated on both complete agar and on minimal agar the heterocaryotic spores formed colonies on both agars while the homocaryotic spores, which in this case were auxotrophic, formed colonies only on complete agar. On minimal medium only 33 per cent of the plated spores formed colonies and on complete medium 35 per cent of the colonies could be recognized as heterocaryotic because of their greenish color, easily distinguishable from the yellow or white of homocaryotic colonies. The sample of spores plated contained 30 per cent heavily stained conidia. Hence, it is quite certain that these heavily

staining conidia are the heterocaryotic ones and that the staining property, the large size and the large number of nuclei are autonomous characters.

We must, then, account for the fact that heterocaryotic conidia are larger and have more nuclei than homocaryotic conidia. It cannot be due to heterocaryotic vigor, for this hypothesis would predict that the wild type homocaryotic conidia should be as large or larger. Nor is there any known reason to suppose that there



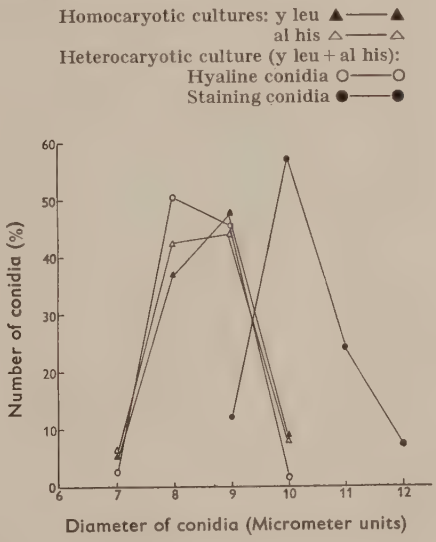


Figure 1 (*left*). The relation of conidial diameters to nuclear number. Data include both staining and hyaline conidia from a heterocaryon (y leu + al his) of *Asp. sojae*. The numbers refer to the numbers of spores found in each class.
Figure 2 (*right*). Distribution of conidial diameters in samples from homocaryotic and heterocaryotic cultures of *Asp. sojae*.

are more diploid nuclei in heterocaryotic conidia than in homocaryotic conidia; moreover, their frequency is only about 10^{-7}. Nor could it be due to the fact that conidia with more nuclei are more likely to be heterocaryotic, for in the homocaryotic strains such highly multinucleate conidia should also be found. Rather it seems that the nuclei contained in the conidial bud may determine when the conidium is constricted from its source. In the heterocaryon this time may depend upon the presence of the proper proportion of the two types of complementary auxotrophic nuclei and this may require that on the average more nuclei be present. Hence the volume of cytoplasm would be increased. Or the metabolism of cytoplasm in which a complementary set of nuclei is present may differ from that of regions that are homocaryotic and depend on an external supply of growth factor. Genetic blocks, such as would exist in homocaryotic sterigmata, are known to alter metabolism in many ways in

addition to preventing specific biosynthesis [9]. In this way the conidia budded off may be larger and have a different staining reaction, which may only result from an accelerated maturation.

Whatever the mechanism, it seems clear that even in the same species, *Asp. sojae*, the constancy of the DNA-cytoplasmic ratio may be maintained by either of two events, reduction of nuclear number or increase of cell volume.

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THE EFFECT OF REMOVAL OF THE JELLY COAT ON FERTILIZATION IN SEA URCHINS

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It was recently shown that the jelly coat surrounding the sea urchin egg to a certain extent acts inhibiting on fertilization [3]. When the eggs were treated in acid sea water at a pH of about 5.8 a considerable improvement in fertilization was brought about upon complete removal of the jelly layers, as measured with the "fertilization rate method" [3, 4, 7, 10]. Under certain conditions the removal of the jelly layers also brings about an increased number of fertilized eggs [4, 10]. The addition of jelly solution in high concentration to eggs with intact jelly layers causes a decrease in the fertilization rate. This impairing action of jelly solution is still more obvious when jelly-free eggs are used [3]. The influence of jelly solution on homologous and heterologous fertilization was studied in detail [3, 4] and it was found that a very dilute jelly solution enhances the fertilization rate which is of special importance in cross fertilization experiments. The action of jelly solution on fertilization is rather nonspecific and similar results have also been obtained with e.g. heparin [4]. The inferences drawn from these results are in contradiction to the "fertilizin theory" [13, 14, 19].

According to the fertilizin theory it is not possible to fertilize fertilizin-free eggs [13, 14], i.e. eggs deprived of their jelly coats (cf. [19, 21]). Later it was found that the removal of the jelly did not completely abolish fertilization, though it was claimed that jelly-free eggs had a lowered capacity for fertilization [18].

The results referred to above [3, 4] suggested an entirely different action of the jelly coat in fertilization than that proposed by the modified fertilizin theory [18, 19]. Therefore it seemed desirable to investigate the susceptibility of the eggs to different concentrations of sperm before and after removal of the jelly coats.

Material and methods.—The experiments were carried out during the period February–May 1955 at Stazione Zoologica, Naples. Eggs and sperm from the sea urchins *Paracentrotus lividus*, *Psammechinus microtuberculatus* and *Arbacia lixula* were used. To remove the jelly coats the eggs were subjected for 60–120 seconds to sea water acidified to pH 5.8. After this treatment the eggs were thoroughly washed in filtered sea water [3]. To make sure that the eggs were completely jelly-free a sample from every batch was stored in a small volume of sea water for 2–3 hours. The supernatant sea water was then tested for its sperm agglutinating capacity [3]. No sperm agglutination was ever observed under these circumstances. Microscopical examination of the eggs before and after staining with Janus green did not reveal any traces of the jelly coat adhering to the egg surface after treatment at pH 5.8. The sperm was obtained by mixing the concentrated sperm from four animals and the number of sperm was counted in a Bürker chamber after fixation in 3–4 per cent formaldehyde.

In each experiment eggs from only one female were used in order to obtain a material as homogeneous as possible. However, there are considerable differences between different animals with respect to fertilizability and the material also changes noticeably from day to day [cf. 12]. Also within every batch of eggs different groups of eggs have been recognised [12]. It must therefore be assumed that the sperm is characterized by similar differences. Consequently, the appropriate concentration range of sperm has to be determined for every material. It is also important that the number of eggs is the same, especially when insemination of intact eggs and jelly-free eggs are to be compared. In all 44 experiments carried out, the results were entirely uniform.

In the experiments the egg suspensions were divided into two parts, one of which was treated with acid sea water [3]. The two lots of eggs were inseminated at the same time with serial dilutions of sperm.

Results.—The results show that eggs deprived of their jelly coats need lower concentrations of sperm to become fertilized to 100 per cent than do eggs with intact jellies. Moreover, the concentration of sperm which fertilized 50 per cent of the eggs was also much lower for jelly-free eggs than for control eggs.

When the concentration of sperm was increased the jelly-free eggs showed polyspermy at much lower concentrations than did eggs with jelly coats. However, if the concentration of sperm was increased to about 10^8 per ml the eggs with jelly coats showed as high incidence for polyspermy as did the jelly-free eggs. This was obviously due to the instantaneous dissolution of the jelly coats in such high concentrations of sperm. A typical experiment with *Paracentrotus* eggs is shown in Table I.

It has also been observed [16] that addition of $NaIO_4$ counteracts the polyspermy

TABLE I

Eggs and sperm from *Paracentrotus lividus*. In each inseminated batch 300 eggs were counted.

Concentration of sperm per ml	Eggs with intact jelly coats		Jelly-free eggs	
	Fertilization %	Polyspermy %	Fertilization %	Polyspermy %
6.5×10^7	100	46	100	63
1.3×10^7	100	8	100	41
2.5×10^6	100	1	100	20
9.0×10^5	100	0	100	15
5.0×10^5	100	0	100	1
1.6×10^5	80	0	99	0
3.0×10^4	26	0	89	0
1.0×10^4	9	0	52	0
3.0×10^3	1	0	13	0
1.0×10^3	0.6	0	9	0

which frequently occurs in underripe sea urchin material from the Swedish west coast ([5], cf. [7]). It was recently indicated [2] that $NaJO_4$ enhances the cortical propagation which would be expected also to speed up the formation of the hyaline layer and consequently the block to polyspermy [1, 6, 8, 9].

In order to investigate whether addition of $NaIO_4$ could also protect the egg against polyspermy if high sperm concentrations vere used, four series of parallel experiments were set up in which control eggs, jelly-free eggs, control eggs pretreated with $NaIO_4$ (10^{-5} molar for 10 minutes), and jelly-free eggs pretreated with $NaIO_4$ (10^{-5} molar for 10 minutes) were subjected to the same sperm concentrations. However, in the series with control eggs treatment with $NaIO_4$ was found to cause a rapid swelling and dissolution of the jelly coats. Consequently, the capacity of the jelly coat for inhibiting polyspermy was more or less abolished and it was difficult to evaluate the results. The jelly-free eggs which show a higher incidence of polyspermy than do the intact eggs (cf. Table I) became less susceptible to polyspermy after pretreatment with $NaIO_4$ in a concentration of $\sim 10^{-5}$ molar. The percentage of polyspermy in jelly-free eggs after such pretreatment was found to be the same as in the untreated control. The incidence of polyspermy in pretreated controls with jelly coats was not markedly changed, because the favourable influence on the cortical reaction exerted by $NaIO_4$ was counteracted by the swelling of the protecting jelly coat.

The removal of the jellies did not produce any negative changes in membrane formation, cleavage and subsequent development. On the contrary it was observed that jelly removal as a rule brought about an evident improvement in membrane elevation (cf. [3]) and the membranes became more highly elevated.

Discussion.—It was previously shown that addition of jelly solution interferes with the membrane formation [15, 17]. In recent investigations it was reported that

a complete removal of the jelly coat at an appropriate pH results in an improvement in fertilization [3, 4, 10]. Moreover it was demonstrated that removal of the jelly coat makes, in some species, the eggs susceptible to heterologous fertilization [4, 11].

In the present investigation the jelly coat was also removed according to previous methods [18, 19], and the eggs were treated at pH 3.5-4.5. This treatment was found to depress the percentage of fertilized eggs, and the membrane formation and the development were also markedly impaired (cf. [20]).

The results reported in this paper show that complete removal of the jelly coat at pH 5.8 does not cause any injury to the egg; membrane formation, cleavage and development are quite normal or even improved upon such treatment. Furthermore, the eggs give 100 per cent fertilization at much lower concentrations of sperm than do the control eggs with intact jellies.

The results also show that the jelly coat protects the eggs against polyspermy. This block to polyspermy is certainly very important, since the establishment of the definite block to polyspermy, which is correlated with the growth of the hyaline layer, requires a certain period of time [2, 6].

It was previously shown that the jelly coat *in situ* presents an obstacle to fertilization [3]. In some species it also represents a block to heterologous fertilization [4, 11]. In this paper it has been demonstrated that the jelly also constitutes a block to polyspermy. It is thus obvious that the jelly coat *in situ* represents a general obstacle to sperm entrance.

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A STAGE MOIST-CHAMBER-MICROMANIPULATOR

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It is a common experience with students using conventional micromanipulators and techniques [1, 2] that situations frequently are encountered unexpectedly during the course of an experiment when the service of a third or fourth manipulator unit of even limited function would be welcome. The instrument illustrated (Fig. 1) not only meets this supplemental need nicely but can be used in a wide variety of ways alone.

The instrument basically is a moist chamber of special design [3] which is equipped with two built-in, manually operated manipulators. With the exception of the manipulator arms (Fig. 1, *B*) which are 3″ 20 gauge Yale B-D Lok-Needles, the instrument is made of sheet Plexiglas. The body (*A*) of it measures 2″ × $3\frac{1}{8}$″ × $\frac{7}{16}$″. It consists essentially of three appropriately shaped plates welded together with Plexiglas solvent: a $\frac{1}{16}$″ thick base plate (*C*), a $\frac{5}{16}$″ thick mid-plate (*D*), and a $\frac{1}{16}$″ thick top plate (*E*) to which is welded a coverslip stop (*M*). Each manipulator arm (*B*) extends into the moist chamber area (*J*) through a fan-shaped slit window (*H*) in the end of the mid-plate, through a closely fitting channel (*K*) which passes through a revolving disc (*F*), and a recess (*I*) in the mid-plate between the revolving disc and the moist chamber area. The revolving discs are closely fitted in the mid-plate (*G*) and held snugly between the top and bottom plates. The discs and the manipulator arm channels through them are lubricated with vaseline. An adjustable friction screw (*L*) governs the freedom of the manipulator arm within the disc and another one (*U*) the revolving disc.

To use, the instrument is set-up and prepared as if it were a conventional moist chamber (or depression slide as effected by sealing the open end of the chamber area with Scotch tape) with dissected or cultured tissue mounted by a hanging drop method on 22 × 40–50 mm coverslips sealed above the moist chamber with vaseline. Glass or metallic micro-tools are mounted in the manipulator arms as follows: (1) Micro-tools generally may be mounted by cementing the shaft of the tool into the end of the manipulator arm with an adhesive wax, or, as in the case of platinum wire electrodes or spatulas, the micro-tips fashioned from the wire are provided with extra long shafts which are threaded through the manipulator arms from the chamber side as indicated (Fig. 2, *R*). (2) In the case of micropipettes, the shaft is inserted into the end of a suitable length of polyethylene tubing (I.D. .034″ × O.D. .06″) which has been threaded into the chamber through the manipulator arm from a stationary syringe in the manner indicated (Fig. 2, *R*). A similar arrangement is set up with agar-saline microelectrodes. The up-turned portion of all micro-tips should be long enough to allow the tip proper to be elevated about $\frac{1}{16}$″ above the surface of the top

plate. Three-dimensional movements are accomplished as indicated (Fig. 1, *N*, *O*, *P*) and it is noteworthy that the micro-tips can be completely rotated in the vertical plane.

Obviously, the micromanipulative precision of this instrument is limited because of being manually activated. However, this can be partly compensated for by practice, by increasing the length of the manipulator arms in combination with

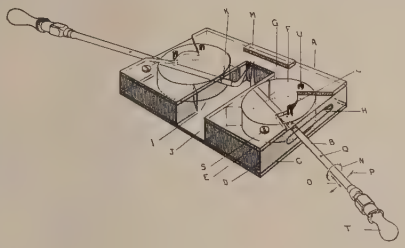

Fig. 1.

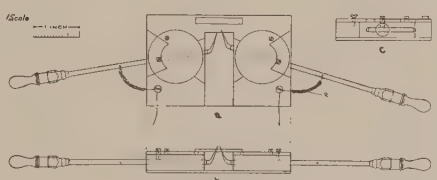

Fig. 2.

control tips (*T*) having greater diameters, and by proper selection of the tasks to be undertaken. This apparatus has been used routinely for a number of years in class-work here, and has had many supplemental and varied uses in connection with studies on the finer structure of striate muscle, on the reaction of cells and tissues in culture to carcinogenes, and in the establishment and handling of single cell clones of protozoa in studies on experimental carcinogenesis [4, 5, 6].

Details for the construction of this apparatus may be obtained by applying the 1″ scale in Fig. 2 to the construction drawings (Fig. 2, *a*, *b*, *c*).

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A HEREDITARY CYTOCHROME DEFICIENCY APPEARING IN YEAST GROWN AT AN ELEVATED TEMPERATURE

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Normal bakers yeast (*Saccharomyces cerevisiae*) spontaneously produces cells ("petites") lacking cytochromes *a* and *b* and thus unable to respire. This metabolic disability is cytoplasmically inherited. The mutation rate to "petite" is greatly increased if the growth medium contains euflavin [1].

An effect similar to that produced by euflavin can be obtained by growing bakers yeast at an elevated temperature. In natural medium (2 per cent glucose, 1 per cent yeast extract, and 1 per cent Bactopeptone), slow growth is barely possible at 40°C. When a normal strain of yeast (showing the bands of cytochromes *a*, *b* and *c*) is grown aerobically at 40°C and the resultant cells are plated out at 30°C, more than 95 per cent of the colonies are "petites" as determined by spectroscopic examination. The "petite" property persists through subsequent transfers at 30°C. Active growth is required to produce this effect. Exposure of normal cells to 40°C in a nitrogen free glucose buffer medium produces no hereditary effect on the cytochrome system.

The above observations do not distinguish between a mutagenic effect of elevated temperature and selection of spontaneously occurring "petite" mutants. Dr. Boris Ephrussi, whose laboratory is continuing the study of this phenomenon, kindly allows me to state that his preliminary results indicate that elevated temperature has a direct mutagenic effect.

In this connection, it is of interest that elevated temperature has been found to induce a hereditary chlorophyl deficiency in *Euglena* [2].

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EVIDENCE FOR A RIBONUCLEOPROTEIN INTERMEDIATE IN THE SYNTHESIS OF GLOBIN BY RETICULOCYTES

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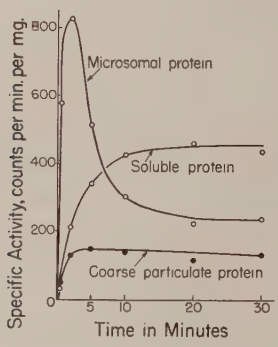
During the past few years of the indirect evidence that ribonucleic acids are controlling factors in protein synthesis [5] has been confirmed by direct biochemical studies with broken cell preparations [8]. Furthermore, *in vivo* tracer studies have indicated that the protein intimately associated with the ribonucleic acid (RNA) of pancreatic [1] and liver [10] microsomes may be precursors of the soluble proteins of these cells. However, subsequent attempts to demonstrate this RNA–protein relationship with individual enzymes of pancreas were not successful [7]. The finding that reticulocytes incorporate radioactive leucine principally into hemoglobin [4] and that the efficiency of this process is correlated with the ribonucleic acid content of the cells [9], suggest the possibility that a ribonucleoprotein precursor to hemoglobin may be found in reticulocytes.

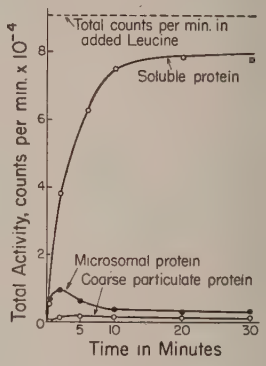
Materials and Procedure. —Rabbit reticulocytes were prepared by daily intramuscular injection for one week of neutralized phenylhydrazine hydrochloride (2.5 per cent w/v) together with folic acid and vitamin B_{12} supplements as recommended by Borsook and coworkers [4]. The cells, obtained by heart puncture, were washed three times by centrifugation at 4°C with cold isotonic saline containing $10^{-3}M$ sodium phosphate, pH 7.2. They were then suspended at a 1:2 ratio in the above medium containing amino acids and iron [3, 4]. These were present in the following concentrations, expressed as molarity $\times 10^4$; L-histidine 0.75, L-valine 3.0, L-phenylalanine 1.6, L-tyrosine 2.0, L-serine 4.0, L-lysine 2.4, L-tryptophan 0.32, L-glutamine 8.5, ferrous ammonium sulfate 1.4.

L-Leucine (7.95 mC/mMole), uniformly labeled with carbon 14, was obtained from the Nuclear Instrument and Chemical Co., Chicago. Solutions of radioactive leucine were prepared in water and added to 4 ml of the cell suspension from a Warburg flask sidearm. The contents of the sidearm were made isotonic by addition of twice isotonic saline-phosphate medium equal to the volume of radioactive amino acid solution.

After temperature equilibration at 37.5°C for 5 minutes, the sidearms were tipped and the incubations carried out for various periods of time. The contents of the flasks were then rapidly mixed 6 ml of cold isotonic sucrose and immediately frozen in a dry ice-cellosolve bath. After partial thawing, the cells were completely disintegrated by exposure for 30 seconds in an iced 9 Kc Raytheon magnetostriction oscillator at full power. The particulate matter was sedimented in 90 minutes at 100,000 $\times g$ in the Spinco ultracentrifuge. It was then resuspended in isotonic sucrose and separated into 2 fractions, a coarse, gray-green material sedimenting in 30 minutes at 1000 $\times g$ and a bright red, translucent microsome-rich pellet sedimenting

in one hour at 100,000 ×g. Both fractions, however, are undoubtedly heterogeneous with regard to the particulate matter of the reticulocyte. Hemoglobin was split by treating an aliquot of the soluble protein with acidic acetone [2]. The colorless protein resulting from the above treatment, mostly globin, and the proteins of the particulate fractions were then washed and counted as previously described [11].





The *in vitro* kinetics of radioactive leucine incorporation into soluble and particulate proteins of rabbit reticulocytes.

Fig. 1 (*left*). Specific activity vs. time. Fig. 2 (*right*). Total activity vs. time. The radioactive leucine contained 90,000 counts per minute in 13 millimicromoles.

RESULTS

The kinetics of the uptake of leucine into the particulate and soluble protein fractions are shown in the figures. Incorporation was essentially complete after 10 minutes when tracer levels of radioactive leucine were used. Fig. 1 shows that during this time the protein of the microsomal fraction had incorporated and subsequently lost radioactive leucine. This loss was not apparent when higher concentrations of radioactive leucine were used, because of the establishment of a steady state. Furthermore, it can be seen in Fig. 2 that during the initial stage of incorporation, after the first one-half minute of incubation, the total radioactivity of the microsomal protein was greater than that of the soluble protein. It is also evident that the radioactive leucine can be almost completely recovered in the soluble protein after 10 minutes.

Paper electrophoresis of the soluble protein in both veronal and borate buffers indicated that the radioactivity of this fraction was associated only with hemoglobin. The marked initial incorporation of radioactive leucine into the microsomal protein may, therefore, be considered to reflect a step in the formation of hemoglobin.

Analysis of the alcohol and ether washed microsome-rich fraction by the orcinol reaction (11) indicated a ribonucleic acid content of 20 per cent. When this fraction was first treated with 0.25 per cent sodium desoxycholate in 0.012*M* glycylglycine

buffer, pH 8.0 [10], a portion of its protein was solubilized. The solubilized material contained nearly all the pigment of this fraction. Its protein had a lower specific activity than that of the protein of the whole fraction, when the microsomal material was isolated after a two minute incubation of cells with radioactive leucine. The desoxycholate insoluble material contained protein whose specific activity was 35 per cent above that of the untreated microsomal fraction. It had a ribonucleic acid content of 30 per cent. Further characterization of the subcellular components of reticulocytes and comparative studies with other labeled amino acids are in progress.

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IN-VIVO EFFECT OF COLCHICINE ON ALKALINE PHOSPHATASE OF RAT INTESTINAL EPITHELIUM

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Colchicine has long been known to arrest mitoses in the metaphase, presumably by inhibiting the action of the achromatic spindle [10]. Effects on cellular metabolism in tumours and normal tissues have been investigated [1, 3, 4, 9, 11, 13]. Inhibition of some tumour phosphatases has been met with.

Colchicine-treated animals often react with diarrhoea. The effect on the intestine has, however, been studied only with regard to mitotic alterations, and functional analysis has so far not been performed. The effect of colchicine on the alkaline phosphatase of intestinal epithelium was therefore studied in the cuticular border and the Golgi region where this enzyme is normally demonstrated.

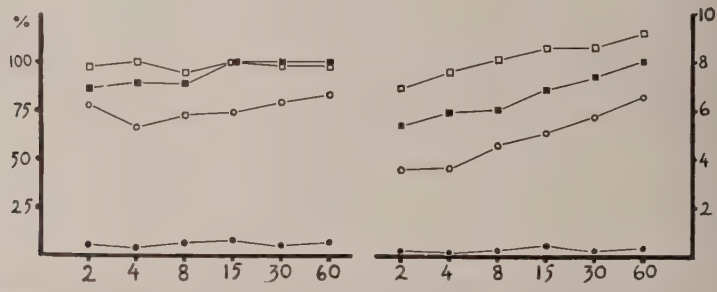

Fig. 1. Mean values of relative areas of alkaline phosphatase stainability in the cuticular border (□, ■) and Golgi region (○, ●) of rat intestinal epithelium (left diagram) and of the intensity of this staining (right diagram). Filled figures indicate experimental animals. Abscissae: incubation periods in minutes.

Eighteen white rats were used for the investigation. Nine of these were given 1 mg of colchicine per kg body-weight subcutaneously and killed after 6 hours. The other nine served as controls. The animals were divided into groups with about an equal number of experimental animals and controls in each.

Pieces of duodenal tissue were fixed in cold absolute acetone and embedded at 55° C in paraffin wax, m.p. 50-52° C. Alkaline phosphatase was demonstrated with a modification [6] of the Gomori method [8], the cobalt technique being used. The incubation periods were 2, 4, 8, 15, 30 and 60 minutes.

In subjective quantitative estimation of a histochemical reaction, some errors are due to simultaneous judgement of entirely different factors, e.g. distribution and intensity. These errors may be considerably diminished if the factors are examined separately. Taking a mean value of the reaction strength over a whole preparation in the common way is by no means satisfactory, since a reaction as a rule does not turn out equally in all the parts concerned. In the present investigation the relative distribution of a certain staining intensity, ranging from + to + + +, was for practical reasons set to maximum 4 points, representing involvement of 100 per cent of the structure in question. Thus, + 2, + + 1 means a weak reaction in 50 per cent and a moderate one in 25 per cent, i.e. a definite reaction present in 75 per cent giving a total of + + + + for the preparation examined. The preparations were always presented to the examiner under code numbers in order to prevent tendentious estimation.

The above principle was applied in order to estimate the mean intensity of staining in the cuticular border and the Golgi region after demonstration of alkaline phosphatase activity. It was found that the reaction in the Golgi region disappears al-

most completely after colchicine treatment while the reaction in the cuticular border decreases (Fig. 1). The differences between the corresponding curves representing staining intensity of the cuticular border and the Golgi region in normal and experimental animals are statistically significant.

The staining intensity is, however, not necessarily proportional to the degree of activity in a certain structure. It is therefore customary to compare histochemical results with biochemical. In biochemical analyses, on the other hand, an actual change in some structure may be of minor influence on a strong total activity, as pointed out recently by Brandes and Bourne [2]. Studying the acid phosphatase of the prostate, they found that the histochemical reaction in the Golgi region was inhibited after castration and stilboestrol treatment, although the change of activity was too small to have been definitely demonstrated with biochemical methods.

The reaction in the Golgi region of intestinal epithelium represents a genuine enzymatic activity [7]. It is evident that the inhibition of activity in the Golgi region could not be connected with the mitosis-arresting effect of colchicine, since only 17.63 per cent of the epithelial cells of rat ileum are found in mitoses under experimental conditions identical with the above-mentioned [12]. There is no reason why there should be a marked difference between ileal and duodenal epithelium in this respect (cf. Leblond and Stevens). Consequently, colchicine not only inhibits mitoses but also somehow interferes with the cellular metabolism during the interphase.

It may be of some interest in this connection to emphasize Foster's [5] observation that the Golgi bodies of parathyroid cells diminished considerably in size after colchicine treatment.

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HEME AND DRY MASS FORMATION DURING RED CELL DEVELOPMENT

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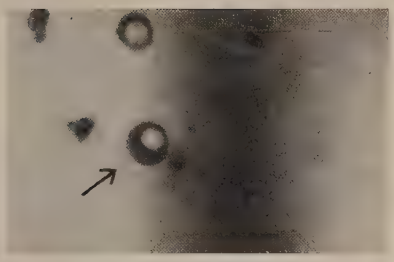
Circulating erythrocytes are developed from the immature erythroblasts through the processes of growth and differentiation. The growth of the erythroblasts involves an increase in the amount of cellular proteins in the presence of cytoplasmic ribose-nucleotides [7]. The characteristic process during the differentiation of the erythroblasts is the formation of hemoglobin which occurs rather late during the development [7]. The hemoglobin consists of two main components, the Fe-containing porphyrin derivative, heme, which as a prostethic group is bound to the protein globin. Incorporation of N^{15}-labeled glycine in regenerating blood and bone marrow of the hen showed that the N^{15}-uptake occurred earlier in the globin than in the heme [6]. This indicated that the maximal rate of the synthesis of the protein component preceded the maximal rate of the prosthetic group formation. Thus, the heme formed during the process of hemoglobinization might be coupled to a previously existing intracellular protein, mainly formed during the phase of growth in the erythroblastic development [9].

This paper deals with the quantitative cytochemical aspects of the above-mentioned relationships between the heme and the protein during the red cell development. The quantity of the cellular proteins is determined as dry mass calculated from micro-interferometric measurements of single erythroblasts. The hemoglobin of the same cells is measured by microspectrography.

Methods.—Bone marrow cells from adult albino rats, weighing about 200 g, were suspended in Ringer solution on a glass slide. A coverslip was put on and the edges sealed with vaseline.

The preparation was then placed in a Dyson interference microscope. The light-source was a Hg-high-pressure lamp with an interference filter for isolating a band at 4900 Å where the absorption effects of hemoglobin are minimal. Condenser: aplanat N.A. 1.3. Interference objective: N.A. 1.3. The microscopical image was photographed (Fig. 1) and the optical phase difference determined in points on the photographic plates by a Spektral-photometer (Zeiss), essentially in the same way as described by Davies *et al.* [4, 5]. The dry mass per unit area was calculated as φ/χ, where $\chi = (\mu_o - \mu_w)/\varrho$ and represents the specific refractive increment. The variation of χ for different proteins is rather small [5] and we have used the common average 0.18. For pure hemoglobin the value of 0.193 has been stated [1, 2].

The hemoglobin of the cell was measured as heme absorption in the Soret-band region of the spectrum (Fig. 2). The same interference microscope setup was used here, but the light filter had been exchanged with a filter transmitting the 4057 Hg line, and the interference objective with an ordinary apochromat N.A. 1.3. The photographically recorded densities in the microscope image were calibrated by



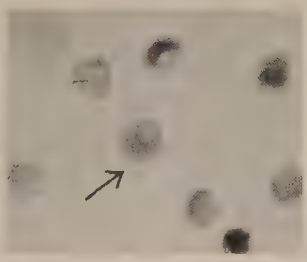


Fig. 1 (*left*). Photomicrograph of an erythroblast suspended in physiological salt solution. Dyson interference microscope, objective of N.A. 1.3, condenser of N.A. 1.0.
Fig. 2 (*right*). Absorption photomicrograph of the same erythroblast as in Fig. 1. Wavelength 4057 Å, objective of N.A. 1.25, condenser N.A. 1.0.

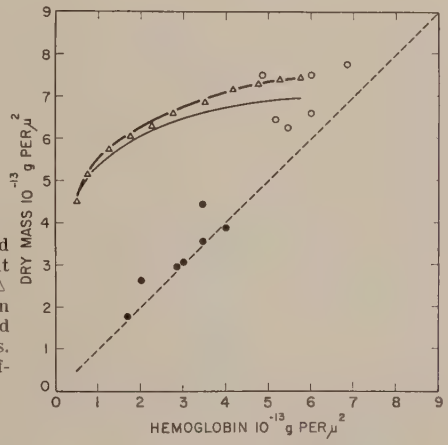


Fig. 3. The relationship between dry mass and hemoglobin per unit area in erythroblasts at various stages of development. The curve △ — △ represents the successive mean values (dispersion 1.13×10^{-13} g/μ^2) from 36 cells. The unfilled circles are values from mature erythrocytes. The filled circles represent erythrocytes at different degrees of hemolysis. See text.

means of a step wedge. The amount of hemoglobin per unit area was calculated according to the principles described previously [7, 8].

Results and Discussion.—By means of the measurements described above, an erythroblast at a certain stage of development could be defined according to the two variables: (1) the cytoplasmic dry mass per unit area, and (2) the cytoplasmic amount of hemoglobin (heme) per unit area. Provided the two main components of the hemoglobin molecule are synthetized *de novo* at the same time, the erythroblastic development would in these coordinates be represented by a straight line. If, however, the protein part, which constitutes the bulk of the dry mass of hemoglobin, partly is present in the cell prior to the heme formation, a curve approaching a value for pure hemoglobin in the mature erythrocytes would be obtained.

For comparison and standardization, a model system was made, using mature erythrocytes at different degrees of hemolysis. Such cells, displaying a variable hemoglobin content, were obtained by immersing the erythrocytes in hypotone salt

solutions. As more than 90 per cent of the mature erythrocytes consists of hemoglobin one might expect a linear relationship between the dry mass and hemoglobin at the different stages of hemolysis. The straight line in Fig. 3 shows that such is the case.

The results from measurements of erythroblasts at various stages of hemoglobinization, on the other hand, show that the main increase in cytoplasmic mass occurs during the earlier stages of the cell development, prior to the final hemoglobinization. The two curves in Fig. 3 are plotted as the successive means from measurements of 36 different cells. In the upper curve $\chi = 0.18$ is used in the calculation of the cytoplasmic dry mass from the phase difference values, in the lower curve $\chi = 0.19$. Both curves approach the value for mature, intact erythrocytes.

Microspectrographic measurements in the ultraviolet spectral range have shown an almost pure "protein band" in the cytoplasm of cells which start to form the heme compound [7]. The nucleotides are too low in concentration to be measurable microspectrophotometrically. Thus, the dry mass changes found during hemoglobinization represent changes in the level of cytoplasmic proteins. Relative to the cytoplasmic heme, this level is much higher in the earlier developmental stages than later. It is reasonable to assume that these proteins are more or less directly utilized for the hemoglobin formation in accordance with earlier findings [9], which of course does not exclude a certain protein synthesis also in the latest stages, i.e. the reticulocytes [3], although this is of minor importance from a quantitative cytochemical point of view.

Summary.—Dry mass and heme absorption have been determined in erythroblasts at different developmental stages. The bulk of dry mass as a quantitative expression for the proteins was mainly formed prior to the synthesis of heme compounds.

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THE INTERFEROMETRY OF HEMOLYSING ERYTHROCYTES

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The amount of hemoglobin in single red blood cells can be studied by micro-absorptiometry [5], phase contrast microscopy [2], or micro-interferometry [1]. The first method is based on the specific absorption of the red blood cell pigment, while in the interference method the total cell mass can be estimated [1, 3].

In hemolysis induced by hypotonic salt solutions or by lysins, more than 90 per cent of the hemoglobin is released from the erythrocytes. Since hemoglobin constitutes more than 90 per cent of the total cell solids, hemolysis produces a change of approximately 85 per cent in the non-aqueous mass of the cell. Large differences in the optical retardation have been demonstrated between intact red cells and ghosts [4]. Such a difference seemed to offer considerable advantages in the determination of the rate of hemoglobin liberation.

Method.—A Dyson interferometer microscope together with a ciné camera were used to record the changing optical retardations of hemolysing human erythrocytes.

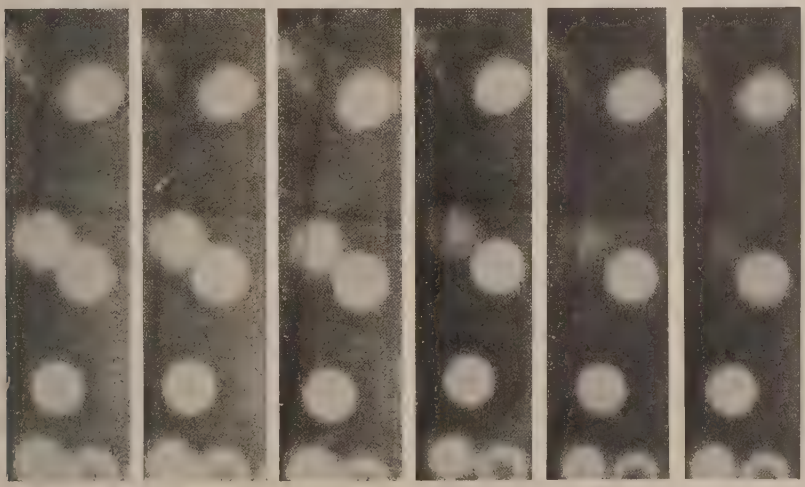

Fig. 1. Successive photographs of the same cells. Only one cell, which gradually fades is seen during hemolysis, and hemoglobin liberation occurred in approximately 7 seconds. The figures below the photographs denote the time in seconds at which the exposure was made after the first picture. (Dyson Interferometer Microscope.)

Essentially monochromatic light of wavelength 546 mμ was used, and an optical retardation of one wavelength corresponds to 3×10^{-12} gμ^{-2}. Erythrocytes were suspended in a fluid containing Michaelis Veronal-Acetate Buffer (10 m Eq), KCl (5 m Eq) and NaCl (150 m Eq). Hemolysis was produced either by introducing small quantities of digitonin or teepol into the cell suspension when it was *in situ* on the microscope, or photodynamically after the addition of Rose Bengal ($10^{-4}M$) to the medium. This last method seemed to be the simplest and most easily controlled way of producing hemolysis. It was possible to record photographically at speeds of 32 frames per second (exposure = $\frac{1}{80}$ second) although 8 frames per second (exposure = $\frac{1}{20}$ second) appeared to be rapid enough to permit analysis of the mass changes. The films were processed in the laboratory under standard conditions. By photometry of this film a curve can be constructed relating change in mass as a function of time.

Great variation in the sensitivity of individual erythrocytes to the lysing agents was noted. Frequently in the case of two almost contiguous cells hemolysis would occur in one, while the other retained a disc like shape. In the figure, which shows one cell hemolysing, the neighbouring cells did not hemolyse for many minutes, and then, at irregular intervals.

The duration of hemolysis varies considerably, and seems to be related in part at least to the concentration of lysin. The maximum time of hemolysis was approximately 12 seconds.

It is hoped to publish a fuller account of these experiments later.

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